The Effects of Mycotoxins on Pregnancy and Immune Development

(A preclinical approach)

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The Effects of Mycotoxins on Pregnancy and Immune Development

(A preclinical approach)

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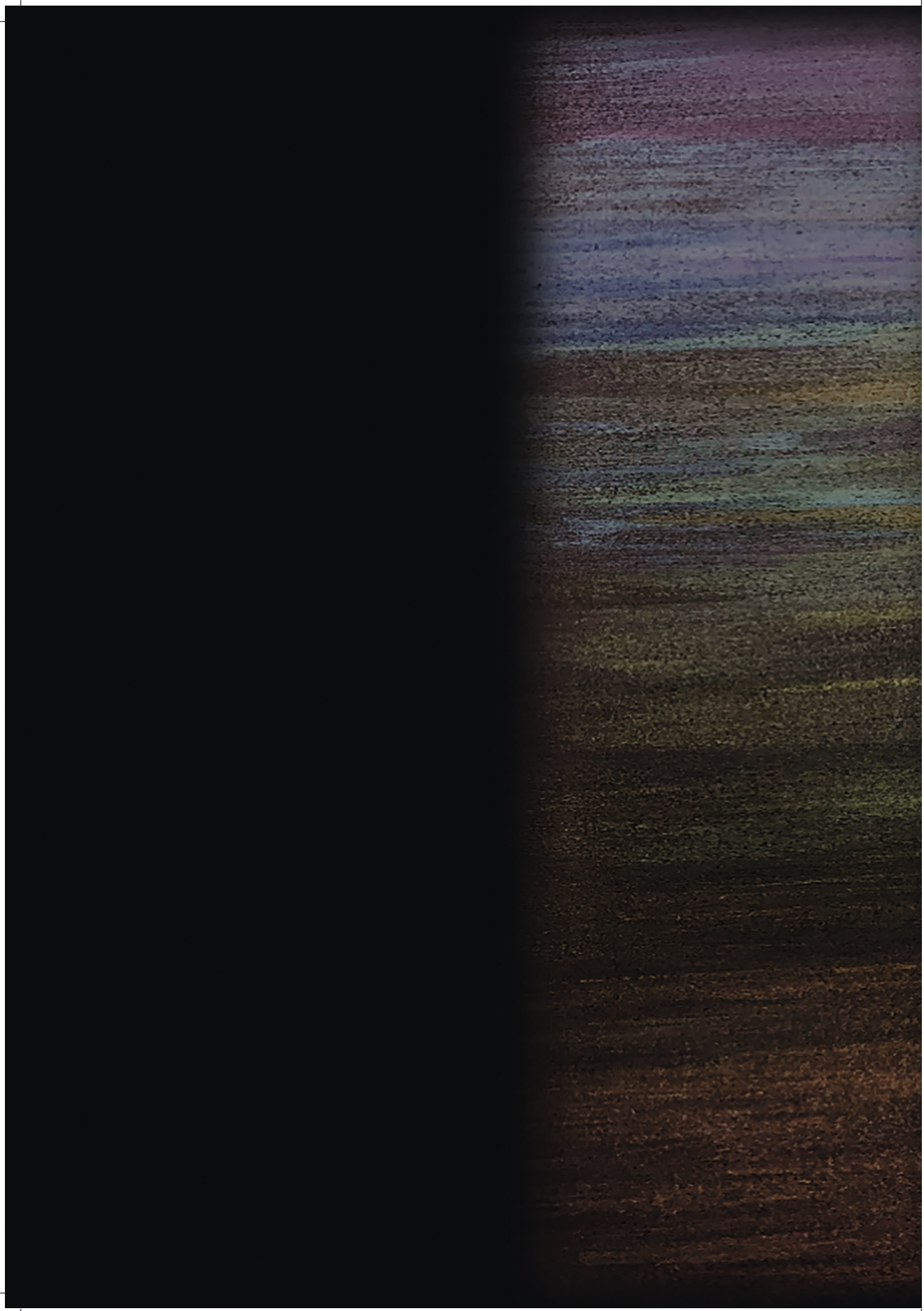

Chapter 1

Introduction: Fusarium Mycotoxins, Impact on Reproduction and Immunity

Mycotoxins

Mycotoxins are among the most important and highly prevalent nutritional contaminants worldwide [1]. They are naturally produced as secondary metabolites of different fungal species, which can contaminate a wide range of agricultural products, especially cereal and grain-based food [2]. Exposure to mycotoxins can occur via the gastrointestinal tract by consuming contaminated food, or via the respiratory tract by inhaling spores of air-born fungi species [3]. More than 300 different mycotoxins have been identified so far, but the most important and prevalent mycotoxins worldwide are produced by fungal species of the Aspergillus, Fusarium and Claviceps genera [4].

Despite all efforts and legislations to limit fungal contamination, human exposure to mycotoxins is inevitable, as contamination with toxigenic fungi species regularly occurs in food supplies at a global level [5]. Furthermore, these toxic products remain stable during food processing steps; therefore, exposure is difficult to prevent [6]. Whether contamination occurs and to what extent, highly depends on climate factors like humidity and temperature on the field or during the harvesting and storage periods. Moreover, masked or metabolized mycotoxins are of great concern in risk assessments because they are hardly detected by commonly used analytical methods, thus leading to underestimation of the total mycotoxin content of food products and thus exposure [7]. Ingestion of mycotoxin contaminated products can lead to acute and/or chronic toxicities in the gastrointestinal tract, nervous system, reproductive organs and the immune system in humans and animals [8]. As exposure is unavoidable, it is essential to explore the health effects of different mycotoxins in humans in detail [9] in order to find more effective strategies to prevent and/or treat mycotoxin-induced toxicities.

Fusarium mycotoxins: prevalence and toxicity

Fusarium species are among the most prevalent fungal contaminants of grains. Over the past decades, Fusarium contamination of crops has become one of the most serious problems in food supplies worldwide and is attributed to climate change and modern agricultural practices [10]. The climate factors such as temperature and moisture, play a key role in mycotoxin contamination. Thus, climate change is expected to further increase the risk of Fusarium contamination in agricultural products [11], which indicates a pressing need to study the health effects of mycotoxins. Two most important classes of mycotoxins produced by Fusarium species are trichothecenes (including deoxynivalenol (DON), T-2 toxin and HT-2 toxin) and zearalenones (ZEN) [12]. According to the BIOMIN World Mycotoxin Survey in 2020 [13], the mycotoxins which are most prevalent globally are Fusarium mycotoxins, DON being the most common contaminant occurring in 65% of crops collected from 79 countries all over the world. Investigating the occurrence of Fusarium mycotoxins and their modified forms in cereal grains originating from different European countries shows a considerable level of contamination exceeding

the maximum tolerable daily intake (TDI) levels [14]. There is limited information about the exact serum levels of these mycotoxins in different populations [15, 16], as the exposure levels may vary based on the source, type and the amount of the grain-based products consumed by every individual.

Both in humans and animals, exposure to Fusarium mycotoxins is associated with carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, neurotoxic, hepatotoxic, and immunotoxic effects [17, 18]. Toxicities of DON, T-2 toxin and ZEN on main biological systems and organs, as well as developing embryo and fetus are summarized in table 1.

DON exposure exhibits intestinal, neurological, reproductive and immunotoxicity [19]. Although DON is reported to be the least lethal trichothecene, acute toxicity with DON leads to severe emesis (therefore, it is also called vomitoxin), anorexia, diarrhea, and abdominal stress [20, 21]. In animal models, chronic and prolonged ingestion of DON-contaminated food caused decrease in weight gain, anorexia and nutritional deficiencies [19]. The mechanisms by which DON causes toxicity are induction of oxidative stress, mitochondrial dysfunction, DNA fragmentation and a ribotoxic stress response [22-25]. Exposure to DON elicits proinflammatory responses, cytotoxicity and apoptosis in different organs [26-28], which can result in growth impairment and impose adverse effects on intestinal epithelium and immune response, immune system regulation and even reproduction [29].

DON-contaminated cereals may contain its acetylated derivatives, 3-acetyl-DON (3ADON) and 15 acetyl-DON (15ADON) as well These fungal derivatives of DON are generally considered equally toxic as DON, though some studies reported higher toxicity of 15ADON on intestinal epithelium, compared to DON [30, 31]. DON can be metabolised to de-epoxy-DON (DOM-1) as well by intestinal microbes [32]. Data concerning the microbial derivatives of DON are scarce and the effects of these modified mycotoxins are not well established, however some studies showed a reduced toxicity of DOM-1 on intestinal tissue, compared to DON [33], but immunomodulatory properties of DOM-1 were similar to those caused by DON [34].

T-2 toxin, a type A trichothecene produced from Fusarium fungi, is one of the most acutely toxic members of the trichothecene family [35]. HT-2 toxin, a major metabolite of T-2 toxin in the body, is considered equally toxic as its parent molecule [36]. T-2 toxin can cause emesis, diarrhea, lethargy, weight loss, hemorrhage, immunotoxicity, necrosis, damage of cartilage tissues, apoptosis, and cell death [35, 37]. It is a well-known inhibitor of protein synthesis through its high binding affinity to the 60s ribosomal subunit [38]. Furthermore, T-2 toxin inhibits the synthesis of DNA and RNA, interferes with the metabolism of membrane phospholipids, and increases the level of liver lipid peroxides [35].

Another highly prevalent Fusarium mycotoxin, ZEN, is a non-steroidal estrogenic mycotoxin, which can cause reproductive disorders due to its estrogenic properties [39]. ZEN has relatively low acute toxicity after oral administration in different animal models [40], however long-term exposure can cause major health effects. The main metabolites of ZEN in the body, α-ZEN and β-ZEN, show similar estrogenic activities as the parent molecule [40]. ZEN and its metabolites bind to estrogen receptors with high binding affinity and exert estrogenic effects [41]. In addition to the endocrine effects, ZEN can induce cell proliferation, oxidative damage, DNA damage, apoptosis, necrosis and finally cell death [42, 43]. ZEN also exhibits hepatotoxicity, haemato-toxicity, immunotoxicity and genotoxicity [44].

Several studies have reported transport of different mycotoxins, such as DON and ZEN and their metabolites, into the breast milk, indicating a potential early-life exposure to these mycotoxins during lactation [45, 46]. Therefore, understanding the potential toxic effects of exposure to mycotoxins in humans, especially during crucial developmental stages, such as pregnancy and lactation, is necessary to prevent or attenuate the adverse health outcomes in early stages of life.

Effects on the immune system

All major Fusarium mycotoxins can induce immunotoxicity [76]. The immune system is highly sensitive to Fusarium mycotoxins; and exposure to low levels that do not induce explicit clinical mycotoxicosis have been shown to significantly modulate immune responses and are thus recognized as immunotoxic compounds [77, 78].

Exposure to DON induces either immunosuppressive or immunostimulatory effects, depending on the concentration and duration of exposure [79]. Immunotoxicity of DON may be induced through oxidative stress and DNA damage [51], and inhibition of lymphocyte proliferation [52]. Direct *in vitro* exposure of immune cells to DON alters LPS-induced cytokine secretion, chemotaxis, and phagocytosis capacities [80], reduces T-cell proliferation and activation and modulates cytokine production capacity [52, 81, 82]. Exposing dendritic cells (DC) to DON results in morphological changes, reduction in DC maturation and activation markers [83, 84], and inhibition of LPS-induced cytokines such as Interleukin (IL)-10 and IL-12 [85]. Sensitizing mice by intragastric gavage of whey proteins in combination with DON enhances allergic reactions to whey proteins, possibly by disturbing the integrity of the intestinal epithelial barrier and inducing cell stress, resulting in the initiation of Th2 responses and allergic reactions [53]. Moreover, a compromised resistance to enteric and pulmonary reovirus infections was reported after DON exposure in mouse models [54, 55], possibly due to suppression of type-1 IFN-mediated responses.

Table 1. A summary of toxicities induced by DON, T-2 toxin and ZEN in different biological systems and organs.

Mycotoxin	Target	Toxicity	Proposed mechanism
DON	Gastrointestinal system	Severe emesis, anorexia, diarrhea, and abdominal stress	Disrupting gut morphology and permeability, gut changes in gut microbiota composition, mitochondrial dysfunction [25, 47, 48]
	Nervous system	Disrupted neuroendocrine and growth hormone signaling, anorexigenic effects, neuroinflammation	Change in neurotransmitters, abnormal lipid peroxidation, impaired signal transduction pathways, neuronal apoptosis [49, 50].
	Immune system	Enhanced allergic reactions to whey proteins, compromised resistance to reovirus infections	Oxidative stress and DNA damage and inhibition of lymphocyte proliferation, morphological and functional changes in DC [51-55]
	Reproductive system	Reduced reproductive performance, increased the incidence of stillborn	Impairing oocyte maturation, Affecting cytoskeletal dynamics, apoptosis, epigenetic modifications [56, 57]
	Fetus/Embryo	Skeletal deformities such as misaligned or fused neural arch and cervical ribs, and missing or fused sternebrae	Osteoclast differentiation, altered expression of several genes related to bone development [57, 58]
T-2 toxin	Gastrointestinal system	Emesis, diarrhea, lethargy, weight loss, hemorrhage	Apoptosis, necrotic lesions [35, 37, 59]
	Nervous system	Cerebral lesions, anorectic responses, ataxia, vertigo	Oxidative stress, mitochondria dysfunction [60, 61]
	Immune system	Altered thymus development, reduced host resistance to pathogens, diminished endocytosis, and phagocytosis of macrophages	Increasing the mRNA and protein expression, apoptosis, induction of proinflammatory cytokine [62-66]
	Reproductive system	Reduced pregnancy and implantation rates, maternal mortality, and embryonic death	Inhibiting spermatogenesis, decreased estradiol secretion by ovaries [67-69]
	Fetus/Embryo	Fetal brain damage, bone malformations	Single-cell necrosis, oxidative stress, apoptosis, mitochondrial dysfunction, and DNA damage in embryonic cells [69-72]

Table 1. *[Continued]*

Mycotoxin	Target	Toxicity	Proposed mechanism
ZEN	Liver, Kidney	Adverse liver lesions with subsequent development of carcinoma, increased activity of hepatic enzymes, Progressive nephropathy	Estrogenic properties, DNA fragmentation, micronuclei formation [40, 44]
	Immune system	Immunosuppression in the liver, immunostimulatory effects in the spleen	Binding with oestrogenic receptors on immune cells, modifying cytokine production [73, 74]
	Reproductive system	Disturbed maturation of ovarian follicles, premature birth, hypoestrogenism, infertility, reduced sperm counts and serum testosterone and progesterone concentrations, abnormal and nonfunctional ovarian folliclesin female offspring	Binding with estradiol receptors, mimicking endogenous estrogen [40, 44, 75].

Similar to DON, the immunotoxicity of T-2 toxin is time- and dose-dependent; it can induce either immunostimulatory or immunosuppressive effects [86]. At low doses, T-2 toxin acts as an immune stimulant, increasing the mRNA and protein expression of proinflammatory cytokines in immune competent cells, both *in vivo* and *in vitro* [62, 63]. *In vitro* exposure of human monocytes to T-2 toxin interferes with their differentiation into macrophages or DC and diminishes the capacities of endocytosis and phagocytosis in macrophages [66]. High doses of T-2 toxin damage lymph nodes, spleen, thymus, intestinal mucosa, bone marrow and white blood cells, hence playing an immunosuppressive role [86]. T-2 toxin can inhibit lymphocyte proliferation, antigen-specific differentiation and induces apoptosis of lymphocytes [87], thereby reducing host resistance to pathogens [64, 65]. Exposure to T-2 toxin significantly affects thymus development [88]. Exposure of thymocytes to T-2 toxin resulted in decreased proliferation *in vitro* [89]. This indicates a high sensitivity of the thymus to T-2 toxin.

ZEN is also known to be immunotoxic, causing several alterations in immunological parameters [44]. Immune cells such as NK cells, macrophages, T cells, monocytes and B cells express estrogen receptors [90], and can be a target for ZEN toxicity. Feeding piglets a ZEN-contaminated diet causes immunosuppression in the liver [73], while inducing immunostimulatory effects in the spleen [74], mainly by altering the expression of pro- and anti-inflammatory cytokines.

Taken together, these data demonstrate that the immune system is vulnerable to the vast range of immunotoxicities induced by exposure to Fusarium mycotoxins. Considering the increasing evidence supporting the significant role of immunotoxins in the development of non-communicable diseases [91], studying the exact mechanisms involved in immune effects of mycotoxins, as well as their long-term consequences is crucial.

Effects on the reproductive system

Pregnancy is a critical period of physiological changes for both the mother and the fetus, and maternal nutrition plays a crucial role in this window of opportunity to shape the long-term health of the infant [92]. However, relatively high levels of mycotoxins were detected in urine samples collected from pregnant women [93-95], indicating that the developing fetus and newborn can potentially be at risk of mycotoxin-induced adverse health effects. Studies in animal models have indeed demonstrated that DON, ZEN, and T-2 toxin can pass through the placenta and adversely affect the development of the fetus during pregnancy [96-98]. Early-life exposure to toxic environmental factors and contaminants, such as mycotoxins, can interfere with developmental processes, causing long-term or permanent structural or functional changes in the offspring [99]. In a recent study in human subjects, different mycotoxins, including DON, were detected in over 75% of all amniotic fluid samples collected from pregnant women who were at high

risk of carrying a child with a chromosomal anomalies or genetic fetal defects, and 73% of the amniotic fluid samples from fetuses with genetic defects had detectable levels of these mycotoxins [94]. These data stress the importance of studying the adverse effects of mycotoxins in early stages of life, including in fetuses, neonates, and infants.

Deoxynivalenol, ZEN, and their major metabolites are detected in fetal and placental samples of pregnant sows and rats receiving contaminated diets during pregnancy [97, 100, 101]. DON exposure during pregnancy can induce teratogenic effects on the fetus [19], the most prominent consequences being abnormal skeletal development and low fetal survival rate [58, 102, 103]. Prenatal DON exposure in rats significantly increased the occurrence of early and late fetal death and resorbed litters [102]. The most common DON-induced deformities in fetal skeleton were misaligned or fused neural arch and cervical ribs and missing or fused sternebrae [103]. Expression of several genes related to bone development in vertebral bones of 18-day old fetuses were significantly altered due to DON exposure, indicating the teratogenic effects of DON at the genomic level [58]. Moreover, DON exposure may affect the quality of reproductive cells, as feeding a DON-contaminated diet to male and female rates prior to breeding decreased the percentage of breeding success and reduced weight gain and food consumption in female pregnant rats and the average weight gain in pups [104].

T-2 toxin and HT-2 toxin are shown to be strongly toxic to embryos [105]. Prenatal exposure to T-2 toxin induces embryonic/fetal death, fetal brain damage [72], and fetal bone malformations [69]. The main mechanisms of the embryotoxicity of T-2 toxin and HT-2 toxin are oxidative stress, apoptosis, mitochondrial dysfunction, and DNA damage in embryonic cells [69-71]. T-2 toxin exposure in pregnant rats led to decreased expression of lipid metabolism-related genes and increased apoptosis in the liver of dams, placenta and fetal liver [70]. T-2 toxin and its metabolite, have a lipophilic nature, and thus can easily be distributed to the fetal brain. Maternal exposure to T-2 toxin in rats increased the single-cell necrosis in the central nervous system of the fetus [106, 107].

Due to its estrogenic properties, direct exposure to ZEN can induce infertility, expansion of the ovaries and uterus [108], and reduction of progesterone levels in female mice, rats and pigs [40], as well as reduction in sperm counts and serum testosterone concentrations in male mice [109]. Major metabolites of ZEN can also induce hyper estrogenic effects in reproductive organs by binding with estradiol receptors in the absence or low levels of estrogen [110]. Exposing pregnant mice and rats to ZEN adversely affects litter size and pregnancy rate, leading to reduced embryonic survival rate, premature birth, and fetal abortion [75, 111]. Prenatal exposure to ZEN has also been shown to cause long-term adverse effects on the reproductive system of the first-generation female offspring in rats, including abnormal and nonfunctional ovarian follicles [75].

Placenta, a sensitive target for mycotoxin-induced toxicity

During pregnancy, the placenta is the only link between the fetus and the mother. It is also an endocrine organ producing a number of hormones which are necessary for maintaining a healthy pregnancy [112]. Placental epithelial layer, consisting of cytotrophoblasts and syncytiotrophoblast, provides a large surface for maternal–fetal exchange of oxygen and carbon dioxide, nutrients and metabolic waste products [113]. Placental epithelial cell lines, such as BeWo cells, are generally used as an *in vitro* model resembling these cellular layers. Both *in vivo* and *in vitro* studies on placental structure and function have shown that Fusarium mycotoxins induce adverse effects. DON exposure in pregnant mice caused oxidative damage in the placenta by increasing the accumulation of reactive oxygen species, which can lead to embryotoxicity [114]. Hemorrhage with apoptosis of cytotrophoblasts was observed in the placenta of rats and mice exposed to T-2 toxin [88]. ZEN exposure to pregnant rats, and BeWo cells *in vitro,* inhibits mRNA expression of the estrogen receptor and altered the expression of major ABC transporters in the placenta [75, 115, 116]. Exposing BeWo cells to ZEN significantly increases the production of human chorionic gonadotropin, which is essential for trophoblast differentiation, and induces morphological modifications in trophoblast cell layer [115]. These studies indicate the sensitivity of the placenta to mycotoxin-induced toxicity.

The placenta is important as a barrier against pathogens and paracellular diffusion of chemicals and toxins [117]. Therefore, any disruption of the integrity of this layer leads to imbalanced maternal–fetal transportation of hazardous chemicals. Although several studies have already demonstrated the harmful effects of mycotoxins on intestinal epithelial barrier function and proinflammatory responses, data related to the effects of mycotoxins on placental barrier function are scarce. Considering the importance of placenta as the only connection point between mother and fetus, understanding the exact mechanisms of mycotoxin-induced toxicities on placenta is crucial.

Consequences of early-life exposure to mycotoxins on the developing immune system

The most critical events in immune development and maturation occur early in life, especially during pregnancy and lactation periods [118, 119]. Therefore, any immune disturbance at this stage can impose significant long-term consequences for the offspring [91, 120]. Considering the placental transfer of DON, T-2 toxin and ZEN during pregnancy, the developing immune system of the fetus can be exposed to these mycotoxins. However, information regarding specific immune effects due to exposure to these mycotoxins during pregnancy is limited. Intravenous administration of DON in pregnant sows alters mRNA expression of inflammatory cytokines in blood leukocytes of piglets and induces a significant decrease in the population of regulatory T cells

in blood samples 1-3 weeks after birth [121]. Maternal exposure to T-2 toxin induces fetal thymic atrophy in mice [89, 122], and exposure to ZEN in pregnant sows causes atrophy of white pulp and expansion of red pulp in spleens of piglets. Moreover, ZEN exposure induces lower mRNA expression of cytokines in the spleen, and lowered serum immunoglobulin levels in both sows and piglets [123]. These results highlight that the developing immune system of the fetus is susceptible to mycotoxin-induced harmful effects. However, there are no data available regarding the long-term outcomes of early-life mycotoxin exposure during pregnancy and lactation on antigen-specific immune responses or the development of immune-related diseases.

Can nutritional intervention with non-digestible oligosaccharides prevent some aspects of mycotoxin-induced toxicities?

Specific non-digestible oligosaccharides (NDOs), such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), which are mainly manufactured by enzymatic trans-glycosylation or through bacterial fermentation [124], have prebiotic and immune-modulating properties comparable to those observed for certain human milk oligosaccharides (HMOs). HMOs are considered non-digestible, as the human body lacks the enzymes required to hydrolyze them, thus they are only partially digested by bacteria in the large intestine [125]. Some specific structures, such as 2'-fucosyllactose (2'FL), are detectable in the systemic circulation after oral administration [126, 127]. HMOs are the first prebiotics that infants receive. They are crucial in the development of a healthy immune system in infants [128]. They act through both microbiota-dependent mechanisms, by rebalancing a healthy intestinal microbiota composition [129, 130], and microbiota-independent mechanisms, by directly interacting with epithelial cells and immune competent cells [131, 132]. There are several commercially produced NDO mixtures, such as short-chain GOS (scGOS), which contain different oligosaccharide structures in part comparable to those isolated from HMOs, regarding the functionality [133], and are known to have anti-inflammatory properties on intestinal epithelial cells [134], and have the capacity to attenuate mucosal inflammatory responses and support the Th1/Th2 balance in the intestinal mucosal immune system [133].

Several dietary intervention studies have reported beneficial properties of NDO mixtures [131, 135] as well as HMOs such as 2'FL [136], on improving Th1-dependent responses to an influenza vaccine, by increasing the proliferation of vaccine-specific T-cell and production of antigen-specific Th1-mediated cytokines in splenocytes of vaccinated mice [136]. Moreover, scGOS/lcFOS and 2'FL reduce symptoms of allergic asthma and food allergy in experimental murine models by induction of regulatory responses and modulating the Th1/Th2 balance [137, 138]. Considering the positive effects of NDOs and HMOs on improving adaptive immune responses, nutritional interventions with these specific structures may be an efficient strategy at least in part

to attenuate the detrimental effects of mycotoxins, especially on the immune system and gastrointestinal tract.

Aims and the outline of this thesis

The overall aim of this thesis was to map the potential toxic effects of exposure to commonly occurring Fusarium mycotoxins on the course of pregnancy and immune development during early stages of life and to investigate the possible mechanisms involved. Within this thesis, a special focus was restricted to DON, as this is the most prevalently occurring food contaminant. A schematic overview of the thesis objectives is shown in figure 1.

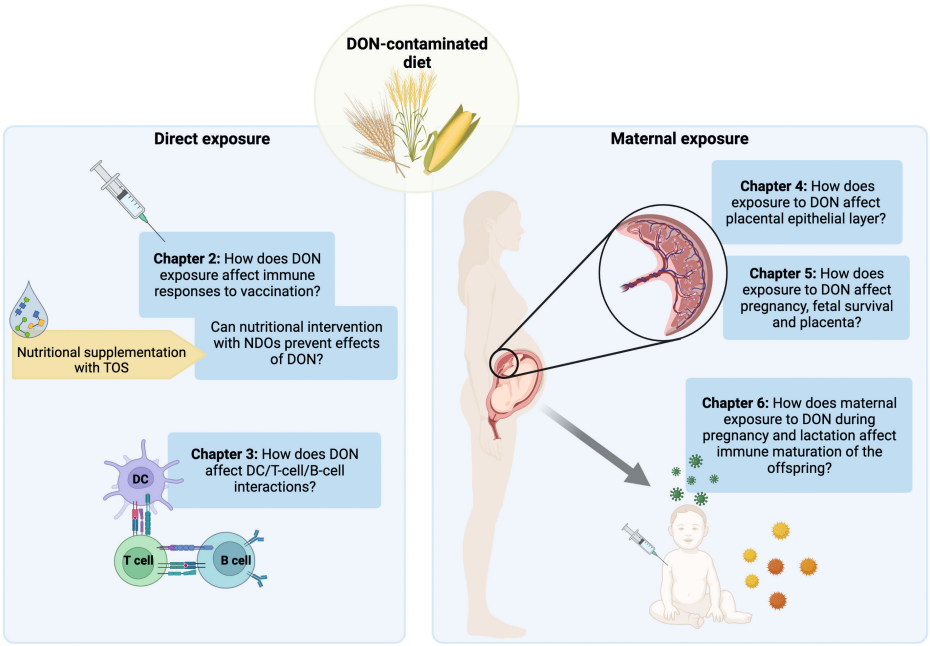

Figure 1. A schematic overview of the thesis objectives and research questions that addressed in each chapter of this thesis. This figure is created with BioRender.com.

Part 1: Impact on immune system

In **chapter 2**, we studied the effects of DON exposure on antigen-specific adaptive immune responses in a validated murine vaccination model. In addition, the possible immunomodulatory effects of dietary supplementation with a specific mixture of NDOs containing high levels of 3'-GL on the vaccine responsiveness in DON-exposed mice was investigated. An in vitro model of the intestinal epithelial cell layer was used to understand the possible mechanisms involved in DON- and 3'-GL-induced changes in the barrier and immune function of these cells.

In **chapter 3**, we developed an in vitro allogeneic coculture model to study the interplay between human monocyte-derived DC, T cells and B cells, and used this model to investigate the effects of DON exposure on DC maturation and activation, as well as on DC-dependent activation and differentiation of naïve T cells and B cells. Furthermore, antigen-specific cytokine production was measured in an ex vivo coculture model using influenza vaccine antigen-loaded murine DC collected from healthy untreated mice and splenocytes collected from vaccinated mice.

Part 2: Impact on pregnancy

In **chapter 4**, the effects of exposure to DON, T-2 toxin and ZEN were investigated using BeWo cells which serve as a suitable in vitro model of the human placental epithelial cell layer. By evaluating the expression and localization of junctional proteins and the expression of pro-inflammatory cytokines, the effects of mycotoxin exposure on placental barrier function and inflammatory responses were assessed.

In **chapter 5**, the consequences of chronic exposure to dietary DON on the course of pregnancy were assessed in a murine model. Changes in the placental barrier and immune markers, as well as the immune modulations in the mother and the fetus were investigated.

Part 3: Long-term effects on immune development

Considering the potential immunotoxicity of DON and its effect on antigen-specific immune responses, in **chapter-6** we studied the effect of maternal exposure to DON during pregnancy and lactation on the immune maturation and Th1/Th2 balance in the offspring using murine models. Ovalbumin (OVA)-induced food allergy model was used to examine the Th2-mediated allergic response, while the influenza-vaccination model was used to assess the Th1-mediated vaccination responses in the offspring of DON-exposed mothers, compared to the offspring of non-exposed control mice.

General discussion and future perspectives

Finally, **chapter 7** includes a general discussion regarding the main findings of this thesis and the overall conclusions. The outcomes of early-life exposure to DON on the course of pregnancy, placental barrier and immune markers, antigen-specific immune responses to influenza vaccine and DC/T-cell/B-cell interactions, and finally the immune maturation of the offspring after maternal exposure to DON during pregnancy and lactation, were discussed. In addition, nutritional interventions with specific NDOs containing 3'-GL were proposed to be beneficial for attenuating and restoring the adverse effects of DON on the immune system.

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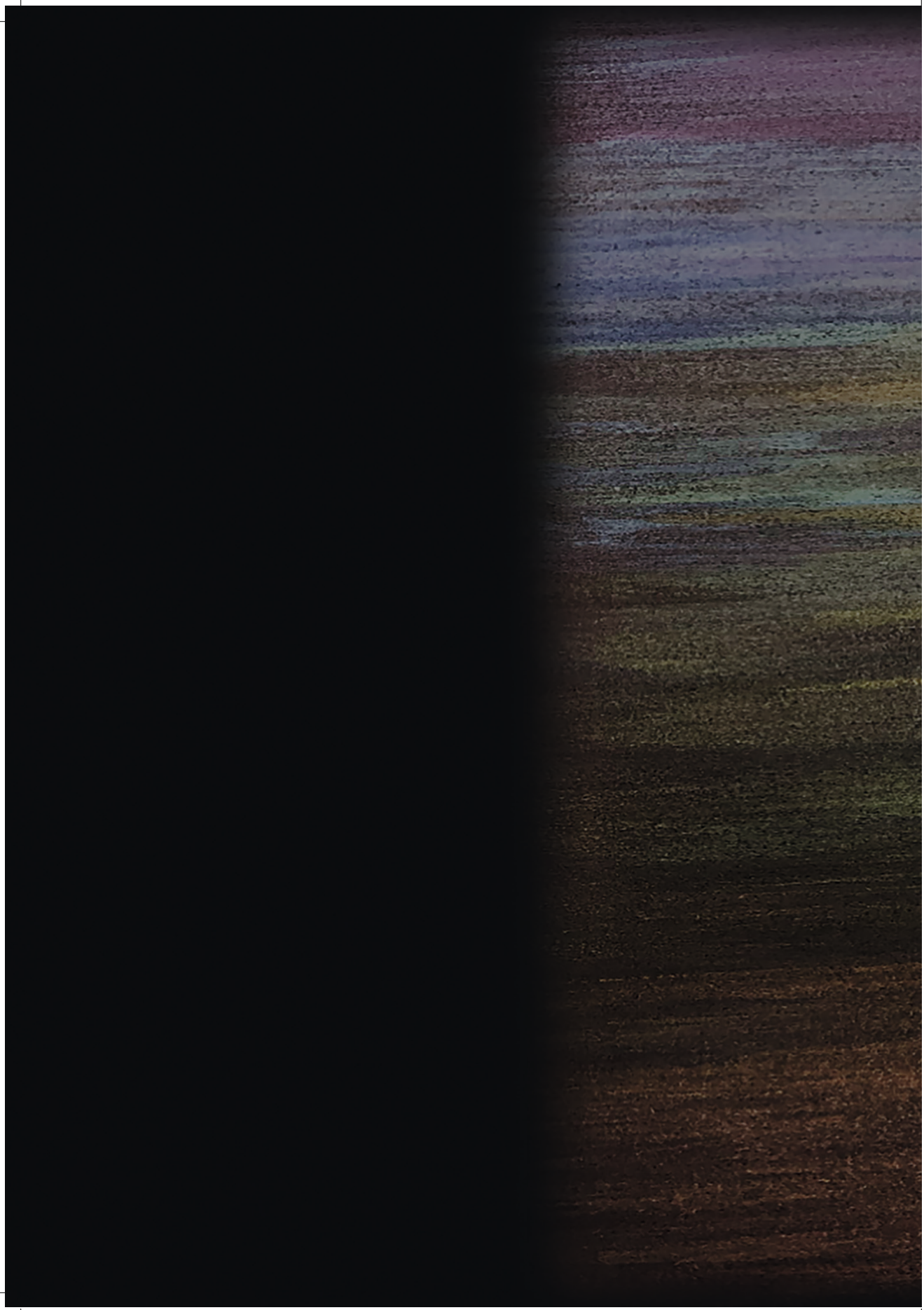

Part 1

Impact on Immune System

Chapter 2

Human milk oligosaccharide, 3'-GL, improves Influenza-specific vaccination responsiveness and immunity after deoxynivalenol exposure in preclinical models.

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Abstract

Deoxynivalenol (DON), a highly prevalent mycotoxin food contaminant, is known to have immunotoxic effects. In the current study, the potential of dietary interventions with specific mixtures of trans-galactosyl-oligosaccharides (TOS) to alleviate these effects were assessed in a murine Influenza vaccination model. Vaccine-specific immune responses were measured in C57Bl/6JOlaHsd mice fed diets containing DON, TOS or a combination, starting 2 weeks before the first vaccination. The direct effects of TOS and its main oligosaccharide, 3'-galactosyl-lactose (3'-GL) on DON-induced damage were studied in Caco-2 cells, as an in vitro model of the intestinal epithelial barrier. Exposure to DON significantly reduced vaccine-specific immune responses and the percentages of $Tbet^+$ Th1 cells and B cells in the spleen. DON significantly altered epithelial structure and integrity in the ileum and reduced the SCFA levels in the cecum. Adding TOS into DON-containing diets significantly improved vaccine-specific immune responses, restored the immune cell balance in the spleen and increased SCFA concentrations in the cecum. Incubating Caco-2 cells with TOS and 3'-GL in vitro further confirmed their protective effects against DON-induced barrier disruption, supporting immune modulation. Overall, dietary intervention with TOS can attenuate the adverse effects of DON on Th1-mediated immune responses and gut homeostasis. These beneficial properties might be linked to the high levels of 3'-GL in TOS.

Introduction

The mycotoxin deoxynivalenol (DON) is a highly prevalent food contaminant, known to induce immunotoxicity in humans and animals. DON is produced as a secondary metabolite from Fusarium fungus species, which contaminates human food at a global level, especially cereal and grain-based products [1, 2]. Acute and chronic exposure to DON have significant negative impact on intestinal, neurological and reproductive systems [3]. The immune system is extremely sensitive to DON, since ingestion of very low levels can alter immune responses [4, 5]. Depending on the concentration and duration of exposure, both immunosuppressive and immunostimulatory effects can be induced upon DON exposure [6]. Higher doses of DON cause immunosuppressive effects, which may be explained by the apoptosis of leukocytes, whereas immunostimulatory effects are seen after exposure to lower doses [7-9]. DON administration in mice decreased the population of antigen-presenting cells and the expression levels of various Toll-like receptors (TLRs) in lymphoid organs, which are critical for immune surveillance [10]. Considering the essential role of the intestinal epithelium in forming a selective barrier between intraluminal dietary antigens and microbes and internal environment, increased gut permeability is associated with different inflammatory diseases and disturbed immune homeostasis [11]. It is already known that DON can damage the intestinal barrier and induce an inflammatory response *in vitro* and *in vivo* and increase the gut permeability [12], whereas the addition of specific non-digestible oligosaccharides (NDOs) such as short-chain galacto-oligosaccharides (scGOS) can protect barrier integrity, mainly by facilitating tight junction assembly and reducing the inflammatory response after DON exposure [13].

Specific NDOs can provide prebiotic and immune-modulating properties similar to those observed for human milk oligosaccharides (HMOs). More than two hundred structurally different forms of HMOs have been identified in breast milk [14]; their concentration depends on several factors, including the stage of lactation and the genetic background of the mother [15]. The structural complexity and diversity of HMOs are unique to human milk. They represent the first prebiotics that infants receive and support both microbiome and immune system development. Although NDOs, as well as HMOs, are only partially digested by bacteria in the intestine [16, 17], some specific structures, such as 2'-fucosyllactose (2'FL) and galacto-oligosaccharides (GOS), are detectable in the systemic circulation after oral administration [18, 19]. HMOs are crucial in the development of a healthy immune system in infants [20]. Various mechanisms have been suggested to explain the immunomodulatory properties of NDOs and HMOs. They are known to be effective prebiotic ingredients and can induce immunomodulatory effects indirectly through microbiota-dependent mechanisms by rebalancing the intestinal microbiota composition and contributing to the development of a healthy intestinal community in infants [21-23]. Moreover, HMOs can induce microbiota-independent immunomodulatory effects through direct interaction with immune

competent cells [24, 25]. Some functional HMO structures, expressed at elevated levels in human colostrum, are based on the elongation of lactose, forming different galactosyl-lactoses (GLs) such as 3'-GL, 4'-GL and 6'-GL [26-28]. There are several NDO mixtures, such as short-chain GOS (scGOS) or trans-galacto-oligosaccharides (TOS), which are mainly manufactured via free enzymatic trans-glycosylation or through bacterial fermentation [29], and contain GLs that are identical to those isolated from HMOs [30]. The composition of those NDOs generated by trans-glycosylation highly depends on the enzyme source and technology chosen. Some specific GLs, such as 3'-, 4'- and 6'-GL, may have anti-inflammatory properties on human intestinal epithelial cells *in vitro*, through inhibition of the NF-κB signaling pathway [27]. Moreover, these GLs have the capacity to attenuate mucosal inflammatory responses during the early developmental stage in intact immature human intestinal mucosa, while supporting the maturation of the intestinal mucosal immune system by modulating Th1/Th2 balance [30]. The beneficial properties of NDOs, improving Th1-dependent vaccine-specific immune responses, have been confirmed in various *in vivo* studies [24, 31, 32]. In order to test for possible similar beneficial effects of 3'-GL on the immune system, TOS was used in the present study, which is another NDO mixture high in 3'-GL (Supplementary Figure S1).

Our group has previously reported that DON facilitates allergic sensitization to whey proteins in mice by disturbing intestinal epithelial integrity and inducing cell stress, resulting in an enhanced initiation of Th2 responses and allergic sensitization [33]. However, information about the effect of DON on Th1-mediated immune responses is scarce. As our earlier observations suggest that DON facilitates Th2-dominated immune responses, which are known to inhibit Th1-mediated immunity, we hypothesized that consuming DON can possibly influence vaccine responsiveness in a murine model. A vaccination model is a well-established and validated method for determining the effect of nutritional interventions on cellular and humoral immune responses, with a focus on Th1-mediated responses [34, 35]. In addition, the possible immunomodulatory effects of dietary supplementation with TOS, containing high levels of 3'-GL (22% wt/wt), were tested by assessing vaccine responsiveness in DON-exposed mice. Moreover, to understand the possible mechanisms involved, and considering the indispensable role of gut microbiome on the development of a healthy immune system [36], the changes induced in intestinal epithelial cell layer and microbial activity were investigated both *in vivo* and *in vitro*. This study provides key insights regarding the potential of dietary intervention with TOS, attenuating the adverse effects of DON on immunity, by improving Th1-mediated immune responses and re-establishing gut homeostasis.

Materials and Methods

In Vivo Experiments

Six-week-old female C57Bl/6JOlaHsd mice were purchased from Envigo (Horst, The Netherlands). Upon arrival, mice were conventionally housed with a light/dark cycle of 12 h/12 h (lights on from 7:00 a.m.–7:00 p.m.) at controlled relative humidity (relative humidity of 50%–55%) and temperature (21 °C ± 2 °C) with access to food and tap water ad libitum. The animals were randomly grouped with 3 mice per cage in filter-topped Makrolon cages (22 cm × 16 cm × 14 cm, floor area 350 cm^2, Technilab BMI, Someren, The Netherlands) with wood-chip bedding (Technilab BMI, Someren, The Netherlands), and tissues (VWR, The Netherlands) and plastic shelters were available as cage enrichments at the animal facility of Utrecht University. The animals received standard diets (pelleted food, AIN-93G, Ssniff Spezialdiäten, Soest, Germany) and routine care for a week upon arrival in the animal facility, before the start of the experiments. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the Utrecht University, and all animal procedures related to the purpose of the research were approved under the license of the national competent authority, securing full compliance with European Directive 2010/63/EU for the use of animals for scientific purposes.

Semi-purified AIN-93G soy protein-based diets were prepared and mixed using different concentrations of DON (FERMENTEK Ltd., Jerusalem, Israel) and/or TOS by Ssniff Spezialdiäten GmbH (Soest, Germany) with concentrations shown in Table 1.

Table 1. Experimental groups, and concentrations of deoxynivalenol (DON) and trans-galacto-oligosaccharides (TOS) in the diets.

Dietary Groups	Vaccination	*n*
Control (AIN93G)	-	3
Control (AIN93G)	+	9
0.5% TOS (wt/wt)	+	9
1% TOS (wt/wt)	+	9
DON (6.25 mg/kg of diet)	+	9
DON (12.5 mg/kg of diet)	+	9
DON (6.25 mg/kg) + 0.5% TOS	+	9
DON (12.5 mg/kg) + 0.5% TOS	+	9
DON (6.25 mg/kg) + 1% TOS	+	9
DON (12.5 mg/kg) + 1% TOS	+	9

TOS was derived via the transgalactosylation of lactose, according to the previously established methods [37], using *S. thermophilus* ST065 beta galactosidase, after which oligosaccharides were obtained via freeze drying (Danone Nutricia Research, Utrecht, The Netherlands). The dry powder contained β3'-GL (22 g/100 g), lactose (45.5g/100 g), glucose (15.6 g/100 g), galactose (5.1 gr/100 gr) and other oligosaccharides (11.7 gr/100 gr). Using high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) the TOS fingerprint was compared to commercial GOS and beta-1,3-galactosyllactose standard (3'-GL, Carbosynth (Berkshire, UK)), as shown in Supplementary Figure S1.

Study Design

After a week of acclimatization, animals were randomly divided into 10 groups (Table 1) and received either control or modified diets. A schematic overview of the experimental design is shown in Figure 1. Vaccination was conducted 2 weeks after starting the diets, using Influvac (Abbott Biologicals B.V., Weesp, The Netherlands) from the 2015/2016 season, as previously described [38]. The mice received the primary and booster vaccinations via subcutaneous injections of 100 µL undiluted Influvac (containing hemagglutinin (HA) and neuraminidase antigens of three strains of myxovirus influenza, in a dose equivalent to 30 µg/mL HA per strain, in total 90 µg/mL HA) in a skin fold of the neck. The booster vaccination was given 21 days after the primary vaccination. The sham-treated group (n = 3, negative control) received injections of 100 µL PBS instead of the vaccine in order to demonstrate the specificity of the vaccine-induced response.

The animals were weighed before starting the diets (day 14) and before booster vaccination (day 21). The weight gain was calculated using the formula:

(weight on day 21) - (weight on day 14) = weight gain (g)

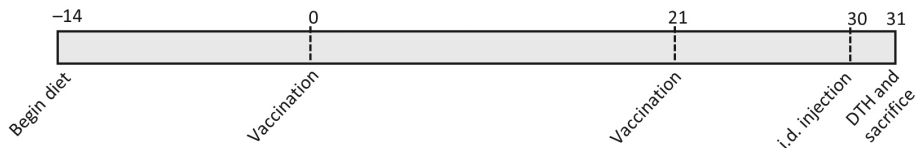

Figure 1. A schematic overview of the murine model of vaccination.

Antigen-Specific Delayed-Type Hypersensitivity Reactions

Delayed-type hypersensitivity (DTH) reactions were induced 9 days after booster vaccination via an intradermal (i.d.) injection of 20 µL Influvac into the ear pinnae of the right ear, and 20 µL PBS into the ear pinnae of left ears as basal line, under isoflurane-induced anesthesia. Ear thickness was measured in duplicate using a digital-micrometer (Mitutoyo Digimatic 293561, Veenendaal, The Netherlands) before injection and 24 h thereafter. The antigen-specific DTH responses were calculated using the formula:

(right ear (thickness at 24h - thickness at 0h)) - (left ear (thickness at 24h - thickness at 0h) = DTH (µm)

The measurements were performed in randomized order and blinded.

Vaccine-Specific Immunoglobulins (Igs) in Serum

Blood was collected at the end of the experiment through orbital extraction under inhalation anesthesia induced by isoflurane, and then cervical dislocation was applied. Blood samples were centrifuged (10,000 rpm for 10 min) to collect the serum and were stored at −20°C until analysis. To determine serum concentration of vaccine-specific antibodies, enzyme-linked immunosorbent assay (ELISA) was performed as described previously [39]. Serum samples were incubated in 96-well plates (Costar EIA/RIA plate, Alphen a/d Rijn, The Netherlands) pre-coated with 1:100 diluted Influvac in PBS. Final dilutions of 1:2000 and 1:8000 of serum samples was used for IgG1 and IgG2a measurements, respectively, and a dilution series of pooled serum that contained vaccine specific antibodies was added for standard curve calculation [38]. For blocking nonspecific binding, the plate was incubated for 1 h with 2% BSA (Sigma, Zwijndrecht, The Netherlands) in PBS at room temperature. Anti-IgG1-biotin and anti-IgG2a-biotin (Becton Dickinson, Heerhugowaard, The Netherlands) antibodies were diluted 1:1000 in dilution buffer (PBS with 0.5% BSA and 0.1% Tween). The plates were subsequently incubated with a 1:20,000 dilution of streptavidin-HRP (Biosource, Etten-Leur, The Netherlands) and optical density was measured with a Benchmark microplate reader (BioRad, Hercules, CA, USA) at a wavelength of 490 nm. Concentrations in test sera were calculated in arbitrary units (AU), relative to the standard curve.

Splenocyte Isolation and Flow Cytometry of Immune Cells

Fresh splenocytes were isolated from spleens using methods described previously [39]. After removing red blood cells by incubating them in lysis buffer (8.3 g NH_4Cl, 1 g KHC_3O, and 37.2 mg EDTA dissolved in 1 L demi water and filter sterilized), splenocytes were counted and resuspended in RPMI 1640 medium containing 10% fetal bovine serum and penicillin (100 U/mL)/streptomycin (100 µg/mL) to reach the concentration of 10^7 cell/mL. Cells were washed in PBS/1% BSA and incubated with anti-mouse CD16/CD32 (1:100 dilution in PBS/5% BSA; Mouse BD Fc Block, BD Pharmingen, San Jose, CA, USA) to block non-specific binding sites. For surface staining, cells were incubated at room temperature for 1 h with CD4 Brilliant Violet 510, CCR6-PE (BioLegend, San Diego, CA, USA), CD69-PE-Cy7, CXCR3-PE, CD25-PerCP-Cy5.5, (eBiosciences, Thermo Fisher Scientific, San Diego, CA, USA) and T1ST2-FITC (MD Biosciences, St. Paul, MN, USA). Viable cells were distinguished by means of a fixable viability dye, eFluor® 780 (eBioscience). For the detection of intracellular transcription factors, cells were first fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol and then stained with Foxp3-FITC (eBioscience), GATA3-PerCP-eFluor710 (eBiosciences, Thermo Fisher Scientific) and Tbet- Alexa Fluor647

(BioLegend) antibodies. Details of the antibodies, including RRID and dilution, are shown in Supplementary Table S1. Results were collected with a BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowLogic software (Inivai Technologies, Mentone, VIC, Australia).

Re-Stimulation of Splenocytes with Vaccine-Loaded Bone Marrow-Derived Dendritic Cells (BMDCs)

Bone marrow cells were isolated from the femurs and tibias of healthy 11-week-old C57BL/6JOlaHsd mice, and were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and Eagle's minimum essential medium (MEM) non-essential amino acids (all from Gibco Life Technologies) in the presence of 10 ng/mL GM-CSF (Prosepec, The Netherlands) for 6 days to obtain immature BMDC (iDC) [38]. Induced iDCs were then loaded with Influvac vaccine at a concentration of 0.9 µg/mL and incubated for 24 h at 37 °C, 5% CO_2 to obtain matured DCs. iDCs treated with medium were used as negative controls. Splenocytes collected from vaccinated mice were cocultured with matured DCs at a 10:1 ratio, in 96-well U-bottom culture plates for 5 days at 37 °C and 5% CO_2, with supplemented RPMI 1640 medium (Gibco).

Cell culture supernatants were collected at day 5 and stored at −20 °C until use and analyzed for the concentration of interleukin (IL)-4, IL-6, IL-10, IL-13, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-2 and interferon (IFN)-γ using a ProcartaPlex multiplex protein assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Isolation of RNA from Intestinal Samples for qRT-PCR

For mRNA isolation, ileum samples (1 cm before the ileocecal junction) collected from vaccinated mice were immediately frozen on dry ice and kept at −80 °C until analysis. Tissue samples were weighed and homogenized in lysis buffer containing β-mercaptoethanol (provided within the RNA isolation kit) with a 1:1 (w/v) ratio, as described before [40]. Total RNA was isolated using spin columns based on the manufacturer's instructions (SV Total RNA Isolation System, Promega Corporation, Madison, WI, USA) and RNA content was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA purity of the samples was confirmed by calculating the 260/280 nm and 260/230 nm ratios. Subsequently, the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to reverse-transcribe the RNA into cDNA, using the T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).

For qPCR, the reaction mixture was prepared by adding selected primers (Bio-Rad Laboratories, Hercules, CA, USA) and iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) to the samples, and amplifications were performed according to the

manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA for each gene was normalized to the expression of GAPDH as a reference, and the relative mRNA expression for each mouse was depicted as a fold change of the average of the control group.

Histomorphometric Analysis of Intestinal Specimens

For histomorphometric analysis, ileum samples collected from vaccinated mice were fixed in 10% neutral buffered formalin in the form of Swiss rolls and embedded in paraffin. The 5-µm sections were stained with hematoxylin/eosin according to standard procedures [41]. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera (magnification of 200×). The morphometric analysis of the sections was performed on 10 randomly selected, well-oriented villi and crypts per animal. A computerized microscope-based image analyzer (Image Pro MC) was used to determine villus height (measured from the tip of the villus to the villus–crypt junction) The villus height was manually defined for each villus separately [12].

Short-Chain Fatty Acid (SCFA) Concentrations in Cecum

Cecum content was collected and immediately frozen on dry ice and stored at −80 °C until analysis. The samples were homogenized in cold PBS and clear supernatants of the homogenates were collected after centrifuging and were stored at −80 °C until analysis. For the measuring of the concentration of acetic, propionic, and butyric acids by gas chromatography, as previously described [42], we used 2-ethylbutyric acid as an internal standard.

In Vitro Experiments

Human epithelial colorectal adenocarcinoma (Caco-2) cells were obtained from American Type Tissue Collection (code HTB-37, Manasse, VA, USA, passage 90–102). Beta-1,3-galactosyllactose (β3'-GL) was obtained from Carbosynth (Berkshire, UK) and alpha-1,3-galactosyl-lactose (α3'-GL) was obtained from Elicityl (Crolles, France). Purified deoxynivalenol (DON) (Sigma Aldrich, St Luis, MO, USA) was dissolved in pure ethanol and stored at −20 °C. TOS was obtained as described above by freeze-drying the sugar products of *S. thermophilus* ST065 beta galactosidase (Danone Nutricia research, Utrecht, the Netherlands).

Cell Culture

Caco-2 cell monolayers were grown in a trans-well system, which is a model for studying intestinal barrier function [43]. Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin was used as culture medium and cells were seeded at a density of 0.3×10^5 cells into 0.3 cm^2 high pore density (0.4 µm) inserts with a polyethylene terephthalate membrane (BD Biosciences, Franklin Lakes, NJ, USA) placed in a 24-well plate. The Caco-2 cells were maintained in a humidified atmosphere of 95% air and 5%

CO_2 at 37 °C. After differentiation of intestinal epithelial cells measured by transepithelial electrical resistance (TEER) exceeding 400 Ω cm^2 (Millicell Electrical Resistance System volt-ohm-meter, Millipore, Temecula, CA, USA), the interventions were initiated. The monolayers were then pretreated for 24 h with 0.25% or 0.5% 3'-GL, or with TOS at the concentration range of 0.25% to 1% before being exposed to DON, which is known to impair intestinal barrier [12]. Subsequently, DON was used at a concentration of 4.2 µM in complete cell medium and added to the apical side, as well as to the basolateral side of the trans-well inserts for 24 h.

The effect of DON, TOS and 3'-GL on cell viability at concentrations used in this study was evaluated by measuring LDH leakage in the culture medium using the Cyto-Tox 96 nonradioactive cytotoxicity assay kit (Promega Corp., Madison, WI, USA), according to manufacturer's instructions.

Caco-2 Cell Monolayer Integrity Measurement

Measurements of the TEER and lucifer yellow (LY) permeability were conducted to investigate intestinal barrier integrity. TEER measurements were performed as previously described [12] and results are expressed as % of the initial value, as measured prior to the pretreatment.

For the determination of paracellular transport, the membrane-impermeable lucifer yellow (LY) (Sigma, St Luis, MO, USA) was added to a concentration of 16 µg/mL to the apical compartment in the trans-well plate for 4 h, and the paracellular flux was determined by measuring the fluorescence intensity in the basolateral compartment with a spectrophotofluorimeter (FLUOstar Optima, BMG Labtech, Offenburg, Germany) set at excitation and emission wavelengths of 410 and 520 nm, respectively.

Cytokine Production

Within cell culture supernatants, the release of interleukin-8 (IL-8 or CXCL8), which is a typical marker for inflammation, was quantified in the medium of the apical side and the basolateral side of the Caco-2 trans-well inserts in response to the treatments. CXCL8 concentrations were measured by using the human ELISA assay (BD Biosciences, Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical Analysis

All data were analyzed by GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA) using one-way ANOVA, followed by Bonferroni's multiple comparison post hoc test for selected comparisons, and for non-normally distributed and non-parametric data, the Kruskal–Wallis test was performed, followed by Dunn's multiple comparisons test. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered statistically significant.

The required sample size for in vivo studies was calculated based on the DTH data available from previous vaccination experiments [38], using G*Power v3.1.9. The estimated effect size was calculated as 0.65 based on our previous experiments, where an outcome of 10% change in ear thickness is considered the minimum relevant difference. The power was set at 0.9 and α was corrected for the number of relevant comparisons (Supplementary Table S2).

Results

Dietary TOS improved vaccine-specific cellular and humoral responses in DON-exposed mice

There was no significant difference in average weight gain between different dietary groups (Figure 2a). There was a significant antigen-specific response to Influvac after i.d. injection, as determined by assessing the DTH reaction (measured as ear swelling) and vaccine-specific IgG1 and IgG2a levels in the serum of mice fed with the control diet. Interestingly, a significant ($p < 0.05$) difference in the influenza-specific DTH response was detected in mice receiving 0.5% TOS as compared to vaccinated mice receiving the control diet (Figure 2b). Dietary DON contamination induced no significant effect on the DTH response, compared to the vaccinated control group. However, when TOS was added to DON-contaminated diets, the higher concentration of TOS (1%) was required to induce significant vaccine-specific immune boosting effects (Figure 2b).

Vaccine-specific IgG2a and IgG1-antibody concentrations were elevated in all vaccinated mice compared to the sham group. TOS supplementation had no significant effect on the DTH-induced increase in IgG1, but it significantly increased ($p < 0.001$) the serum level of IgG2a at the concentration of 0.5% (Figure 2c, d) in vaccinated mice. Dietary DON contamination induced a significant reduction in the IgG1 level in vaccinated mice ($p < 0.05$ and $p < 0.01$ for the concentrations of 6.25 mg DON/kg and 12.5 mg DON/kg, respectively), whereas it had no effect on enhanced IgG2a level. The addition of TOS to DON-contaminated diets had no effect on the DON-induced reduction in IgG1 production, but it was able to significantly increase the IgG2a level ($p < 0.01$) in animals receiving 6.25 mg DON/kg in their diet (Figure 2c, d).

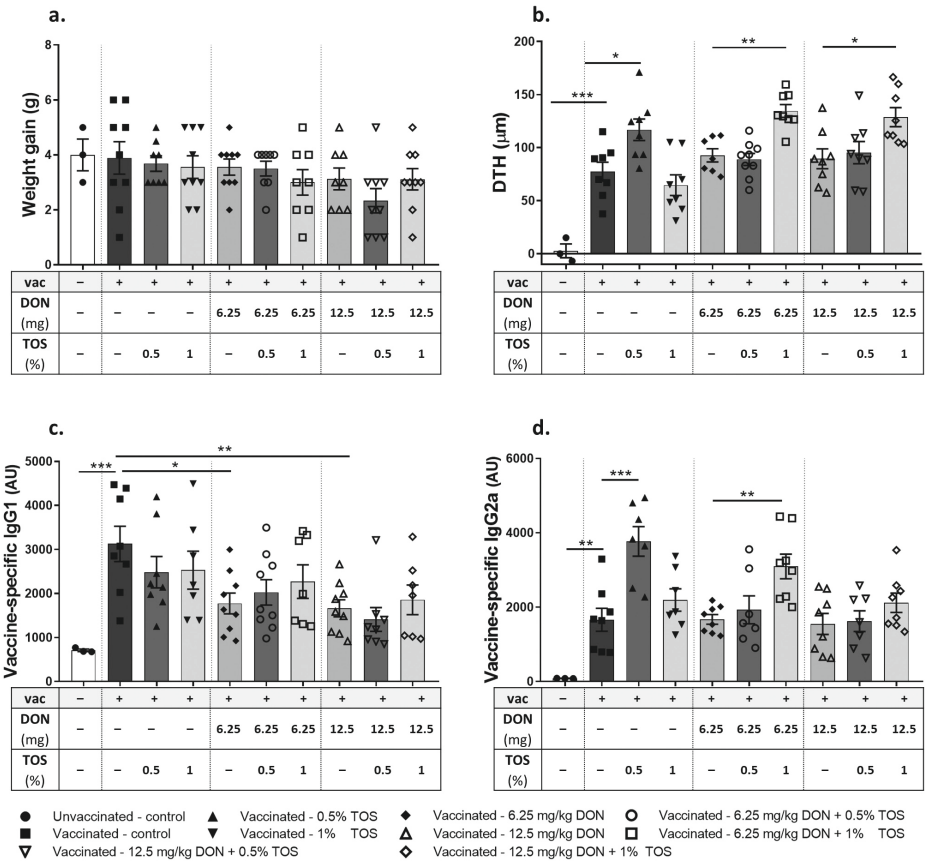

Figure 2. Effect of dietary intervention with deoxynivalenol (DON) and trans-galacto-oligosaccharides (TOS) on weight gain, vaccine-specific delayed-type hypersensitivity (DTH) responses and antibody levels in serum collected on day 31 from vaccinated (vac +) and unvaccinated (vac –) mice. (**a**) Weight gain throughout the experiment; (**b**) DTH response; (**c**) vaccine-specific IgG1; and (**d**) IgG2a levels in serum, measured by means of ELISA assays. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistical differences.

Dietary TOS increased Th1 cell activation and attenuated DON-induced modification in T Cell populations in the spleen

The frequency and activation status of regulatory T cells (Treg) and T helper cells (Th1 and Th2) in isolated spleen samples were studied using flow cytometry (Figure 3, gating strategy). The vaccine-induced DTH reaction did not significantly affect the percentage of $CD25^+$ $FoxP3^+$ Treg cells in the spleen (Figure 4a). In addition, the percentage of these Treg cells was not significantly affected by the addition of TOS or DON into the diet of vaccinated mice, but the addition of TOS to DON-contaminated diets significantly increased the percentage of Treg cells in the spleens of vaccinated mice, compared to the animals exposed only to DON (Figure 4a).

The vaccine-induced DTH reaction did not significantly affect CXCR3$^+$ Th1 cell and T1ST2$^+$ Th2 cell percentages in the spleen (Figure 4b,c). In addition, no significant effect due to the presence of TOS or DON in the diet was observed on the levels of CXCR3$^+$ Th1 cells or T1ST2$^+$ Th2 cells in vaccinated mice. However, both concentrations of TOS increased CD69$^+$ activated Th1 cell numbers compared to those in vaccinated animals in control diet group ($p < 0.05$) (Figure 4d). DON contamination with the higher concentration used in this study induced a significant reduction in Tbet$^+$ CXCR3$^+$ Th1 cells in the spleen of vaccinated mice, compared to controls ($p < 0.01$), but the addition of TOS with both concentrations increased the percentage of Tbet$^+$ Th1 cells in the spleen of DON-exposed mice ($p < 0.05$), and restored it to the values of the control group (Figure 4e).

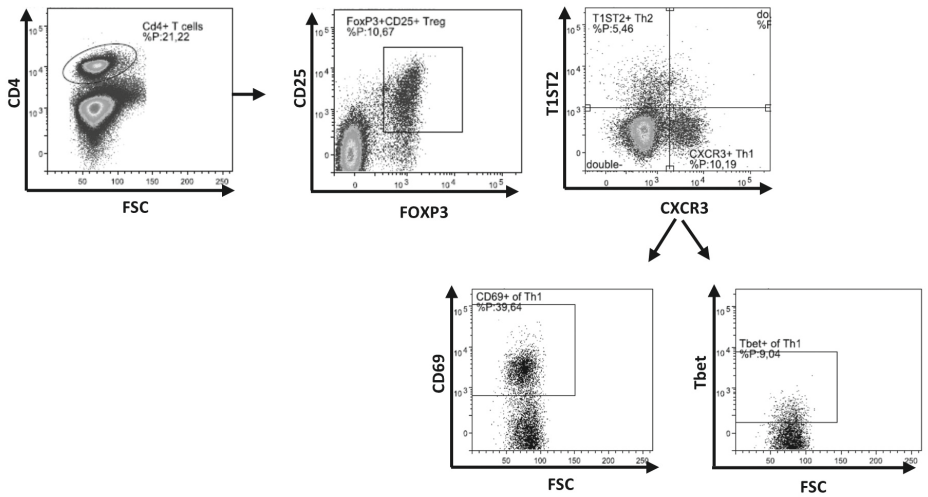

Figure 3. Flow cytometric analysis of T cell subpopulations in spleen. Gating strategy for selecting CD25$^+$Foxp3$^+$ regulatory T cell (Treg), T1ST2$^+$ Th2 cells, CXCR3$^+$ Th1 cells and CD69$^+$ and Tbet$^+$ cells from CXCR3$^+$ Th1 cells.

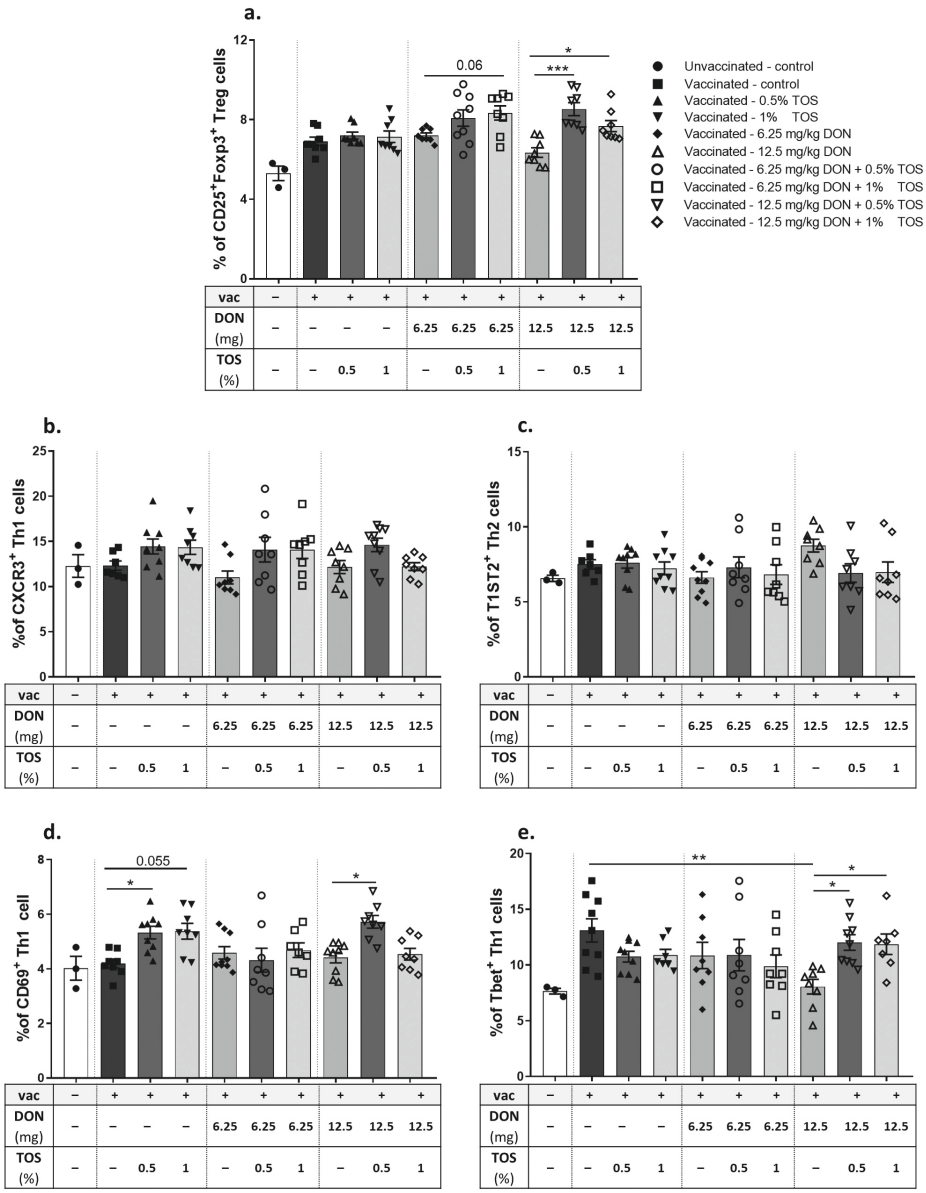

Figure 4. Flow cytometric analysis of T cell subpopulations in the spleen of vaccinated (vac +) and unvaccinated (vac –) mice fed on diets containing deoxynivalenol (DON) and/or trans-galacto-oligosaccharides (TOS). Percentages of (**a**) $CD25^{+}Foxp3^{+}$ regulatory T cell (Treg), (**b**) $CXCR3^{+}$ Th1 cells, (**c**) $T1ST2^{+}$ Th2, (**d**) $CD69^{+}CXCR3^{+}$ Th1 cells and (**e**) $Tbet^{+}$ $CXCR3^{+}$ Th1 cells. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ indicate statistical differences.

Dietary TOS reversed the effect of DON on the B cell population and activation in the spleen

The frequency and activation status of B cells in isolated spleen samples were studied using flow cytometry (Figure 5a, gating strategy). Surface marker expression analysis of CD19 and CD220 revealed no significant effect on $CD19^+$ $B220^+$ B cells in the spleens of vaccinated and control-diet- or TOS-receiving mice compared to the control mice (Figure 4). Both concentrations of DON induced significant reductions in $CD19^+$ $B220^+$ B cell populations in the spleens of vaccinated mice undergoing a DTH reaction ($p < 0.01$). In line with the changes detected in the IgG level, the addition of 1% TOS to DON-contaminated diets significantly ($p < 0.001$) increased the percentage of B cells in the spleen and was able to restore the effect of DON on the DTH-induced B cell response in the spleen (Figure 5b).

CD27 expression was used to distinguish between memory and naive B cells [44]. The DTH reaction had no significant effect on the percentage of memory B cells. Both concentrations of TOS significantly increased the percentage of $CD19^+$ $B220^+$ $CD27^+$ activated B cells in the spleens of vaccinated mice ($p < 0.01$ and $p < 0.05$ for 0.5% and 1% TOS, respectively) (Figure 5c). Interestingly, the percentage of memory B cells was significantly increased in vaccinated mice fed with DON-contaminated diets ($p < 0.01$), but supplementation with 1% TOS in DON-exposed mice reduced the frequency of memory B cells to the values similar to the control groups (Figure 5c).

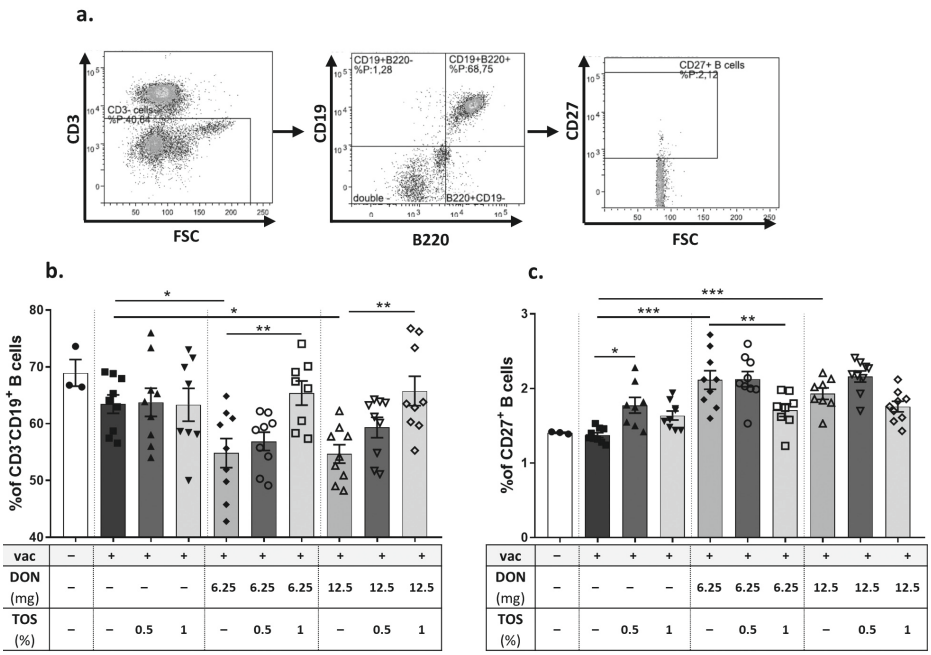

Figure 5. Flow cytometric analysis of B cell subpopulations in spleen of vaccinated (vac +) and unvaccinated (vac –) mice fed on diets containing deoxynivalenol (DON) and/or trans-galacto-oligosaccharides (TOS). Gating strategy (**a**), percentage of $CD19^{+}B220^{+}$ B-cells (**b**) and $CD27^{+}$ memory B cells (**c**). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistical differences.

Dietary TOS increased the production of type-1 cytokine IFN-γ and regulatory cytokine IL-10 from re-stimulated splenocytes of DON-exposed mice

In order to study the cytokine production capacity of immunocompetent cells in vaccinated mice, collected splenocytes were re-stimulated ex vivo by co-culturing the cells with antigen-loaded dendritic cells. Dietary TOS or DON did not induce any significant effect on concentrations of IL-4, IL-6, IL-13, TNF-α or MIP-2 in cell supernatants, compared to control-fed vaccinated mice (Supplementary Figure S2). The production of type-1 cytokine IFN-γ and regulatory cytokine IL-10 from splenocytes was significantly lower in vaccinated mice receiving DON in their diet, compared to vaccinated mice receiving the control diet. Dietary supplementation with TOS had no significant effect on the IL-10 release compared to the control diet but significantly increased IFN-γ release from re-stimulated splenocytes compared to the control-fed vaccinated group ($p < 0.05$) and was able to prevent DON-induced reduction in IFN-γ and IL-10 production (Figure 6). Supplementation with 1% TOS significantly increased IL-10 production in mice receiving 6.25 mg and 12.5 mg DON/kg of the diet ($p < 0.01$ and 0.05, respectively), and increased the IFN-γ level in mice receiving 6.25 mg DON/kg of diet ($p < 0.01$). However, a concentration of 0.5% was more effective in preventing the DON-induced IFN-γ reduction in mice receiving 12.5 mg DO/kg in their diet.

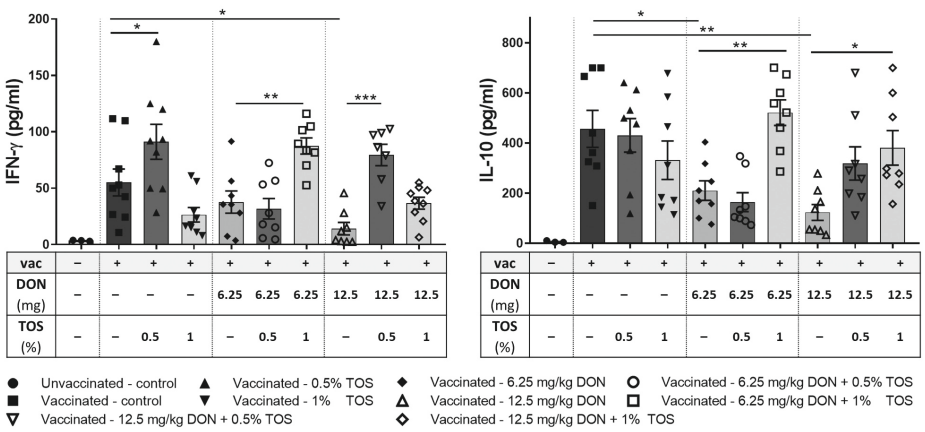

Figure 6. Cytokine production of co-cultured splenocytes of vaccinated (vac +) and unvaccinated (vac –) mice fed on diets containing deoxynivalenol (DON) and/or trans-galacto-oligosaccharides (TOS), with influenza-loaded bone marrow-derived DCs. Interferon (IFN)-γ and interleukin (IL)-10 concentrations in supernatant. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistical differences.

2

Dietary TOS reversed the effect of DON on mRNA expression of junctional proteins and chemokine CXCL1 in the ileum

The ileal mRNA expression profiles of different junctional proteins were analyzed to evaluate intestinal barrier function, which is known to be affected by DON. Feeding mice with 12.5 mg DON/kg significantly reduced the ZO-1 mRNA expression in the ileum of vaccinated mice. Dietary supplementation with TOS upregulated the expression of ZO-1 mRNA ($p < 0.05$) and reversed DON-induced significant downregulation of this tight junction in the ileum ($p < 0.001$ and $p < 0.05$ for 0.5% and 1% TOS in diets containing 12.5 mg DON/kg) (Figure 7a). Although the effect of TOS or DON on E-cadherin mRNA expression was not statistically significant, the addition of TOS to the diet containing 12.5 mg DON/kg significantly increased the expression of E-cadherin mRNA in the ilea of vaccinated mice (Figure 7b). There was no significant difference between groups in the mRNA expression of OCLD and CLDN-3 proteins (Supplementary Figure S3). Furthermore, mRNA expression of chemokines CXCL2/MIP-2 and CXCL1/KC in ileal sections were measured. These chemokines are regarded as functional homologues of human CXCL8/IL-8, and are thus responsible for the initiation of inflammatory cascades and the recruitment of neutrophils into the mucosa [45]. No significant effect was observed on expression of CXCL2 by TOS or DON, but ileal mRNA expression of CXCL1 was significantly increased by 12.5 mg DON/kg in the diet in vaccinated mice compared to the control diet ($p < 0.05$), whereas supplementing DON-contaminated diets with TOS prevented the DON-induced upregulation of CXCL1 (Figure 7b).

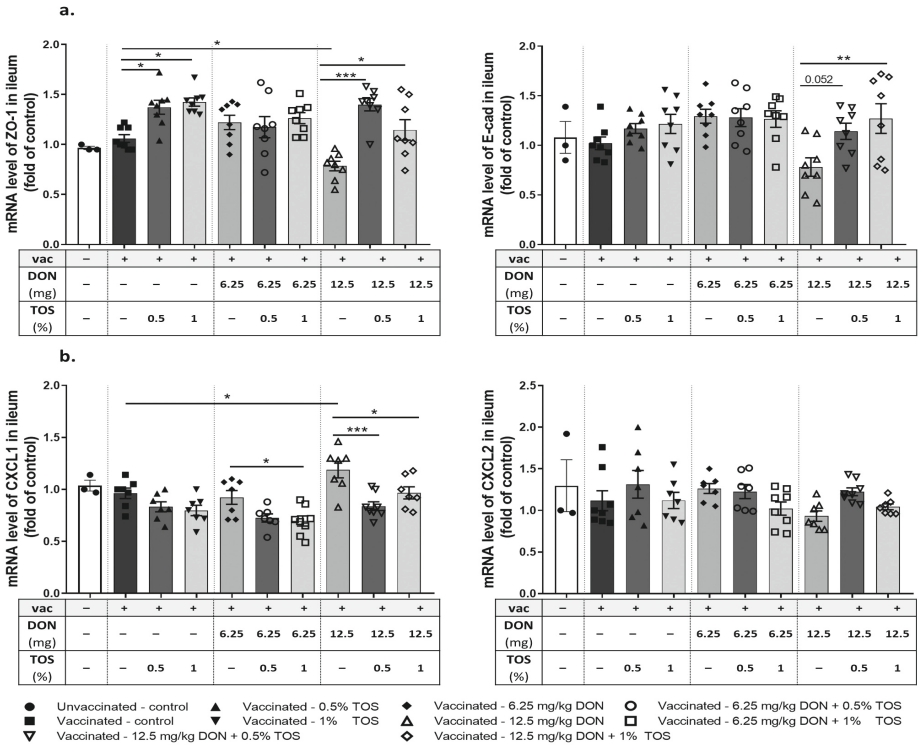

Figure 7. mRNA expression in ileum samples collected from vaccinated (vac +) and unvaccinated (vac –) mice fed on diets containing deoxynivalenol (DON) and/or trans-galacto-oligosaccharides (TOS). Relative mRNA expression of (**a**) tight junction protein zonula occludens (ZO)-1 and adherent junction protein E-cadherin (E-cad), and (**b**) murine chemokines CXCL2 (or MIP-2) and CXCL1 (or KC). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistical differences.

Dietary TOS prevented the adverse effect of DON on villus height of ileum sections
Quantitative histomorphometry analysis was performed on ileum sections of mice fed with control and DON-contaminated diets (12.5 mg DON/kg of diet), with or without 1% TOS. A significant decrease in villus height was observed in the ilea of mice that received 12.5 mg DON/kg in their diet ($p < 0.01$), but the addition of 1% TOS to this diet was able to restore the villus height to normal (Figure 8).

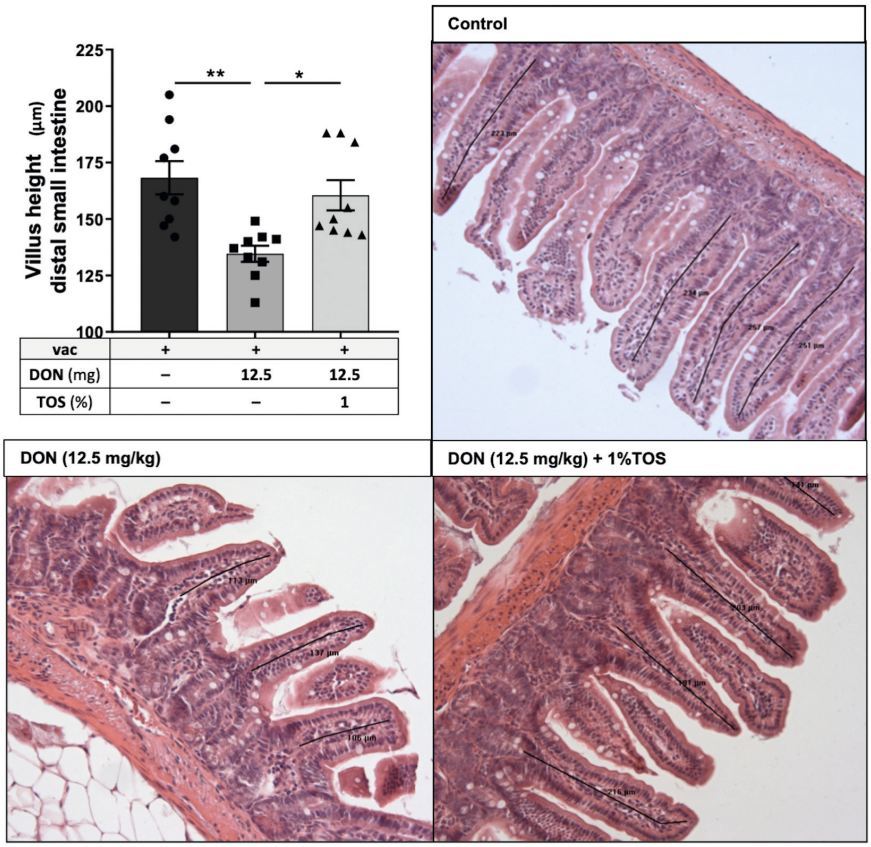

Figure 8. Villus height in ileum sections of the small intestine. Representative photomicrographs of H&E-stained tissue from mice receiving either control or deoxynivalenol (DON, 12.5 mg/kg) ± trans-galacto-oligosaccharides (TOS, 1% wt/wt)-containing diets. Data are presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$ indicate statistical differences.

Dietary TOS had no significant effect on SCFA, whereas a higher concentration of DON reduced SCFA levels in the cecum

Concentrations of SCFA in the cecum were determined as a measure of microbiota metabolic activity to monitor the changes in the microbiota. Dietary TOS supplementation induced no significant effect on concentrations of different SCFAs in vaccinated mice compared to the control diet. However, DON contamination, especially with higher concentrations, caused a significant reduction in acetate, propionate and butyrate productions in mice undergoing a vaccination-induced DTH reaction (Figure 9). TOS supplementation in DON-contaminated diets significantly elevated the acetate level ($p < 0.05$), but its effect on the DON-induced reduction of propionate and butyrate was not statistically significant (Figure 9).

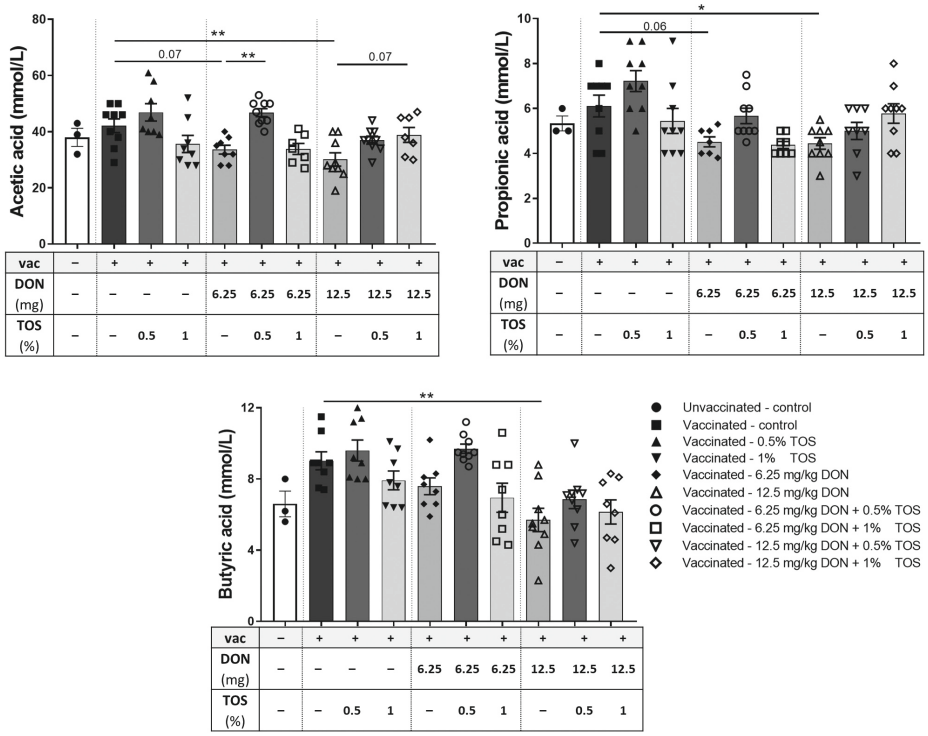

Figure 9. Short-chain fatty acid (SCFA) concentrations in the cecum of vaccinated (vac +) and unvaccinated (vac –) mice fed on diets containing deoxynivalenol (DON) and/or trans-galacto-oligosaccharides (TOS). The concentrations of acetic, propionic and butyric acids were evident in the clear supernatants of cecum contents. Data are presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$ indicate statistical differences.

Protective effect of TOS and its main component, 3'-GL, on DON-induced impairment of Caco-2 cell monolayer integrity and IL-8 production

The direct cytotoxicity of DON and TOS at tested concentrations were measured via the LDH leakage assay. DON at the concentration of 4.2 µM did not impair cell viability, as indicated by the LDH release in the culture medium (Figure 10a). Treating the cells with TOS at concentrations of 0.25% and 0.5% did not affect the cell viability; however, higher concentrations of TOS (0.75% and 1%) significantly increased the LDH release from Caco-2 cells ($p < 0.05$ and $p < 0.001$, respectively). Therefore, lower concentrations of TOS were used in subsequent experiments.

Pretreatment with 0.25% and 0.5% TOS prevented the DON-induced epithelial barrier disruption, as observed by the increase in TEER values and the decrease in the paracellular flux of LY in DON-exposed cells (Figure 10c, d). Moreover, the DON-induced increase in CXCL8 secretion was prevented by preincubation with both 0.25% and 0.5% TOS (Figure 10b).

Since the main content of TOS is 3'-GL, protective effects of pure 3'-GLs on DON-exposed Caco-2 cells were studied. Treating Caco-2 cells with 0.25% of 3'-GL did not affect the DON-induced barrier disruption and CXCL8 production (Figure 11); however, 0.5% 3'-GL could significantly reduce the DON-induced CXCL8 release (Figure 11b) and improve epithelial barrier integrity in DON-exposed cells, as measured through the reduction in the LY flux and the increase in the TEER value (Figure 11c,d).

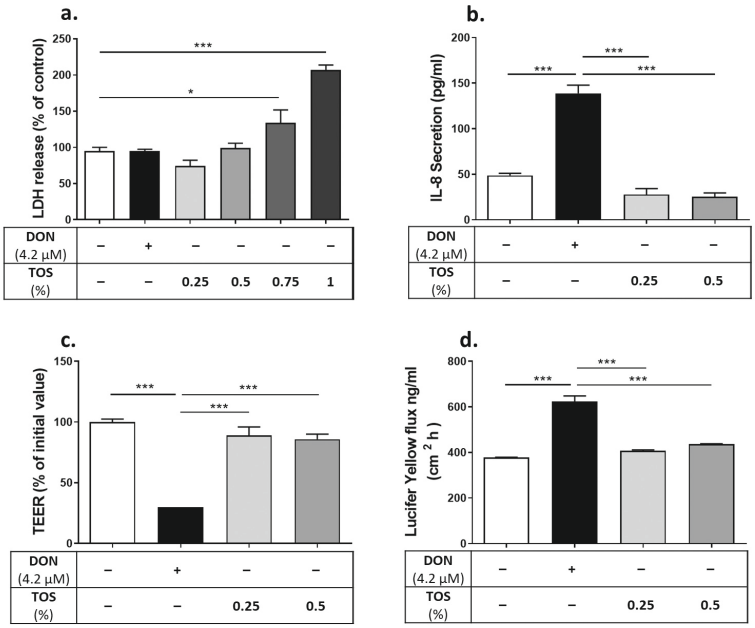

Figure 10. Protective effect of trans-galacto-oligosaccharides (TOS) on deoxynivalenol (DON)-induced adverse effects on Caco-2 cell monolayer. Lactate dehydrogenase (LDH) release in cell supernatant (**a**); IL-8/CXCL8 concentration in cell supernatant (**b**); trans-epithelial electrical resistance (TEER) (**c**) and lucifer yellow flux (**d**). Data are presented as mean ± SEM of 3 independent experiments performed in triplicate. * $p < 0.05$ and *** $p < 0.001$ indicate statistical differences.

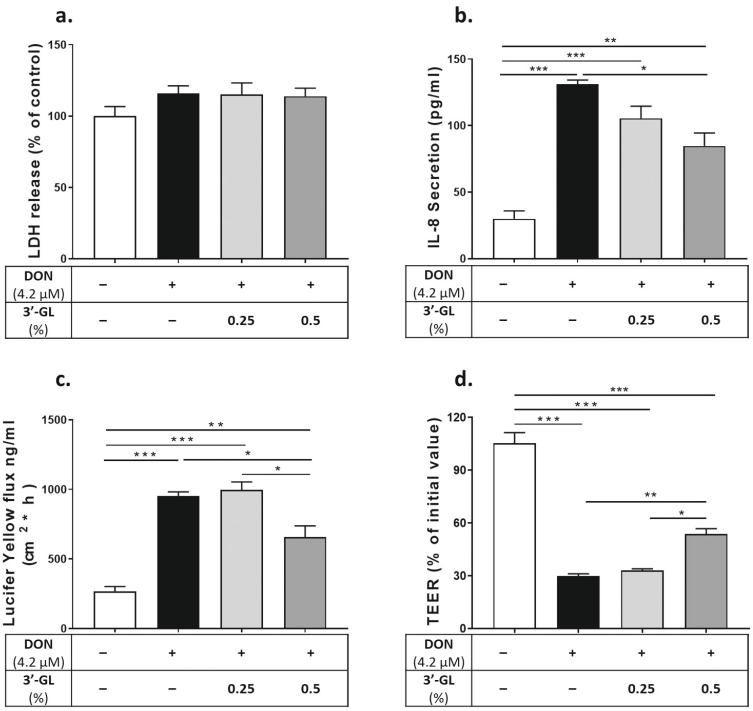

Figure 11. Protective effect of 3'- galactosyllactose (GL) on deoxynivalenol (DON)-induced adverse effects on Caco-2 cell monolayer. Lactate dehydrogenase (LDH) release in cell supernatant (**a**); IL-8/CXCL8 concentration in cell supernatant (**b**); trans-epithelial electrical resistance (TEER) (**c**); and lucifer yellow flux (**d**); Data are presented as mean ± SEM of 3 independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate statistical differences.

Discussion and conclusion

Exposure to many food contaminants, such as mycotoxins, is associated with a wide spectrum of effects on the immune system [46]. Given their high prevalence and stability throughout food-processing steps, understanding the immunotoxicity of these food-associated contaminants is crucial in order to come up with efficient preventive strategies. Here, we investigated the adverse effects of the highly prevalent mycotoxin DON on cellular and humoral immune responses to vaccination against the influenza virus in mice, and showed that dietary TOS, a specific mixture of NDOs containing high levels of GL structures similar to those isolated from human milk, induces immune-boosting effects and protects against DON-induced intestinal and immune compromising effects, both in vitro and in vivo.

Dietary supplementation with 0.5% TOS enhanced Th1-dependent cellular and humoral immunity, as measured through increased DTH response and IgG2a production after vaccination. These data are in line with the higher percentage of activated $CD69^+$ Th1 cells in the spleen and the increase in the production of IFN-γ from re-stimulated splenocytes of vaccinated mice. Previous studies using dietary intervention with scGOS/lcFOS-containing NDO mixtures [24, 31, 32], as well as human milk oligosaccharide 2'-fucosyllactose (2'FL) [38] and the combination thereof [47], have shown similar effects on Th1-dependent responses to an influenza vaccine, and increased proliferation of vaccine-specific $CD4^+$ and $CD8^+$ T-cell and higher production of IFN-γ after *ex-vivo* re-stimulation of splenocytes in vaccinated mice [38]. Different optimal levels of specific NDOs have been observed within this model before. Xiao et al. showed that dietary supplementation with 2'-FL up to 1% increased influenza-specific DTH response in vaccinated mice dose-dependently, whereas higher concentrations of 2'-FL (>1%) did not show the same pattern [38]. In addition, Vos et al. showed that a dietary intervention with scGOS/lcFOS at a concentration of 5%, increased DTH response, whereas higher concentrations did not induce the same effect in a similar model [32]. This clearly indicates that these oligosaccharides can be the most effective at a certain structure-specific optimum level in this model. Enhancing systemic Th1-dependent adaptive immune responses would lead, in theory, to better immune responses against infections and can be beneficial in attenuating the excessive Th2 responses, which occur in allergies. Such effects have been observed for dietary scGOS/lcFOS and 2'FL, as they could reduce the symptoms of allergic asthma and food allergy in mouse models via the induction of $IL\text{-}10^+$ T regulatory cells and modulating the Th1/Th2 balance and suppressing Th2-related parameters [48, 49].

Adding DON to the diet of vaccinated mice had a specifically detrimental effect on B-cell-mediated humoral immunity, as indicated by reduced vaccine-specific immunoglobulin production. Although supplementation with TOS at a concentration of 0.5% could effectively improve vaccine-specific DTH and IgG levels, a higher concentration of TOS

was required to impose similar effects when animals were fed with DON-contaminated diets. DON exposure also decreased Tbet$^+$ Th1 cells in the spleen and induced a significant reduction in IFN-γ secretion from splenocytes after ex vivo re-stimulation. These results are in line with previous observations, where compromised resistance to enteric and pulmonary reovirus infections were reported after DON exposure in mouse models [50, 51]. DON exposure transiently diminished the host response to reovirus by suppressing IFN-γ and increasing IL-4 mRNA expression in Payer's patches and consequently suppressing type-1 IFN-mediated responses [50, 51]. The results of our study showed that addition of 1% TOS in DON-contaminated diets increased the frequency of Tbet$^+$ Th1 cells and the secretion of IFN-γ from re-stimulated splenocytes, and therefore prevented DON-induced reduction in type-1 immune responses in vaccinated animals. Moreover, consuming DON-contaminated diets caused a significant drop in the frequency of B cells in the spleen of vaccinated mice, which corresponds to the reduction in vaccine-specific IgG production in these animals. Interestingly, DON-exposed mice had more CD27$^+$ memory B cells in their spleens, which could be a compensatory response to the reduced antibody production capacity of these cells. The addition of 1% TOS to the diet of DON-exposed mice restored the percentage of B cells to the values of the control group. It can be concluded that TOS supplementation could restore the Th1/Th2 balance, as well as B cells' activity in the spleen and improve vaccination responsiveness in DON-exposed mice.

CD25$^+$ and IL-10$^+$ regulatory T cells have a prominent role in the immunomodulatory properties of some scGOS/lcFOS-containing NDO mixtures, and some HMOs such as 2'-FL and 6'-sialyllactose (6'-SL) are shown to enhance Th1 and diminish Th2 responses [24, 49, 52]. Although the presence of TOS alone in mouse diets had no significant effect on regulatory T cells, the addition of TOS to DON-contaminated diets significantly increased CD25$^+$FoxP3$^+$ Treg cells in the spleens of vaccinated mice. Moreover, dietary TOS increased the regulatory cytokine IL-10 release from the splenocytes of DON-exposed mice. The observed effects of TOS on regulatory T cells and IL-10 could be a reaction to the Th2-skewing effects of DON and could represent a mechanism to restore the Th1/Th2 balance after DON exposure.

Another possible explanation for the immunomodulatory properties of prebiotic oligosaccharides may be that they affect microbiota-dependent mechanisms by rebalancing microbiota composition in the gut [25]. There is a potential link between changes in gut microbial metabolites and improved vaccine-specific immune responses upon HMOS supplementation [47, 53]. Clinical analysis of SCFA concentrations in fecal samples of 3–5 months old infants showed that in exclusively breastfed infants the relative proportion of acetate was higher compared to non-breastfed infants [54]. SCFA profiles produced upon fermentation of HMOs by gut bacteria have well-established anti-inflammatory properties and regulate innate immune cells, such as macrophages, neutrophils and DCs, as well as antigen-specific adaptive immunity, mediated by T cells

and B cells [55]. SCFAs have a significant impact on regulatory T cells and effector T cells by upregulating gene expression during lymphocyte activation and can enhance the mucosal and systemic antibody responses [55]. DON-contaminated diets significantly reduced SCFA concentrations in the cecum content of mice, and this effect was restored through the addition of TOS to the diets. The effect of TOS on SCFA was more prominent in terms of the acetic acid concentration, indicating that TOS enhances specific microbial communities in the gut. Acetate is produced in high levels by certain bacteria, such as various *Bifidobacterium* and *Bacteroides* species, and can enhance mucus production and goblet cell differentiation, thereby supporting the intestinal epithelial barrier and immune function [56]. Moreover, *Bifidobacteria* strains in the human gut are able to utilize 3'-GL efficiently [57]. The prebiotic properties of TOS possibly play an important role in modulating immune responses.

DON is known to disrupt epithelial barrier function and integrity, mainly by disrupting the expression and localization of junctional proteins [12, 58], and it can reduce the villus height in the intestine [12]. In the present study, feeding mice diets contaminated with DON suppressed the mRNA expression of tight junction protein ZO-1 in the ileum, and shortened the height of the villi, which is in line with the observations in previous studies. Shortened villus height could possibly be part of the repair mechanism to overcome the barrier dysfunction after DON exposure by reducing the surface area of the villi [59]. The addition of TOS to DON-contaminated diets upregulated mRNA expression of junctional proteins and restored the villus height in ileal sections to control values in vaccinated mice. Similar beneficial effects have been reported for scGOS as it can attenuate the destructive effects of DON on villus architecture in B6C3F1 mice, and the intestinal barrier of Caco-2 cells, possibly by stimulating the tight junction assembly in the epithelial cell layer [13]. Similarly, administration of 2'-FL in rats had a trophic effect, indicated by higher villus heights and areas in the intestine [60]. The observed effect of TOS could be a result of directly enhancing epithelial barrier maturation and mucus production and modulating the expression of junctional proteins [61] or could be due to the enhanced production of SCFAs, which are known to improve intestinal barrier integrity and protect the mucosal layer [62, 63].

Imbalanced expression of junctional proteins leads to a leaky intestinal barrier and allows antigens to cross the epithelium more easily, resulting in the production of cytokines. This can influence the uptake and processing of foreign antigens by DCs, and therefore influence the development of effector cells from naïve T cells [20]. Murine chemokines CXCL2/MIP-2 and CXCL1/KC are known to be functional homologs of human CXCL8 and have been found to contribute to the initiation of inflammatory cascades and the recruitment of neutrophils into the mucosa [45]. In the present study, DON exposure increased the expression of chemokine CXCL1/KC in ileal sections, which was significantly suppressed by dietary TOS supplementation. Similar anti-inflammatory properties and reductions in CXCL1/LC expression have been reported

for scGOS in DON-exposed mice [13]. The release and synthesis of these chemokines are Toll-like receptor (TLR)4-dependent, and it is known that intestinal epithelial cells are capable of expressing this receptor [64]. There is a significant role for TLR4 in the immunomodulatory properties of different HMOs, such as 2'-FL, and NDO mixtures of scGOS/lcFOS [20, 65]. Therefore, TLR4-dependent pathways may possibly be partly involved in the anti-inflammatory effects of TOS in the intestine. Further investigations are required to fully understand the mechanisms involved in the protective effects of TOS against DON-induced intestinal barrier disruption and inflammation.

To confirm the in vivo observations on intestinal integrity and to study the direct effect of TOS and its main component, 3'-GL, on the epithelial barrier in more detail, single-cell monolayers of the Caco-2 cell line were used as an in vitro model of the human intestinal epithelial barrier. Caco-2 cells are able to fully polarize to form brush borders and cell–cell junctions, and therefore represent the morphologic characteristics of normal human enterocytes [66]. Pre-treatment with TOS prevented the adverse effect of DON on the barrier integrity of the Caco-2 cell layer, and reduced DON-induced CXCL8 production. These results are in line with the in vivo observations in this study and confirm the protective effect of TOS on the intestinal epithelial layer. Similar beneficial effects were observed for 3'-GL on DON-induced barrier disruption and CXCL8 production. However, the observed effects on the Caco-2 cell model cannot be explained by TLR4-dependent pathways, since these cells have very low surface expression of TLR4 [67], indicating that other pathways might be involved in the protective properties of 3'-GL on the intestine.

In conclusion, exposure to DON downregulates immune responses to vaccination through reducing Th1-mediated cellular and humoral immune responses in mice. Dietary intervention with specific oligosaccharides, TOS, can attenuate the adverse effects of DON on the systemic adaptive immune response by restoring the Th1/Th2 balance and improving vaccine responsiveness. It can also re-establish gut microbial activity and protect the intestinal epithelial barrier. The results of in vitro experiments indicate that the observed properties of TOS on gut microbiota and the epithelial barrier are possibly linked to the 3'-GL present in the mixture, which is one of the specific oligosaccharide structures present in human milk. Therefore, dietary supplementation of infant formulae with TOS could be beneficial in boosting the immune system and in the development of a healthy gut microbiome and preventing the harmful effects of food-contaminant DON on the intestinal and immune systems. We also showed that 3'-GL, the main component of TOS, is in part responsible for the observed effects of TOS on the intestine, which signifies the importance of studying the effects of individual oligosaccharide structures on intestine and systemic immune development.

Author Contributions

The author's responsibilities were as follows B.v.L., S.B., G.F. and A.H. designed the study and supervised the data interpretation; J.G., S.W. and A.D.K. supervised the data interpretation; N.S.T., I.v.A., T.L.-M. and T.W. conducted the in vivo experiment; N.S.T. performed data analysis; S.V. and Y.C. conducted the in vitro experiment; S.T. conducted microbial metabolism experiment and data analysis; B.S. made specific contributions to the program with regard to functional oligosaccharides; N.S.T. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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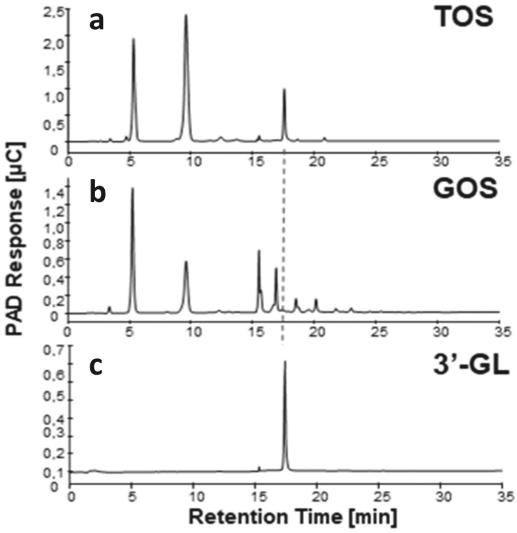
Supplementary content



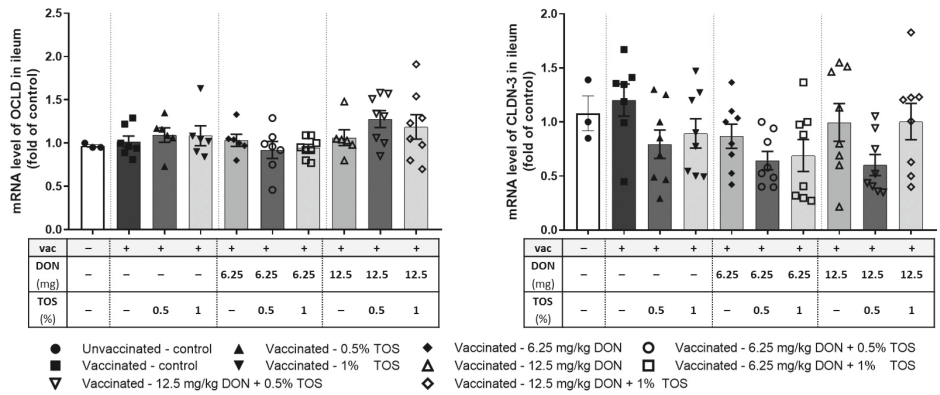
Supplementary figure 1. HPAEC-PAD generated TOS fingerprint (a), GOS (b), Beta1,3-galactosyllactose standard (3'-GL) (c).

Supplementary table 1. List of antibodies used in flowcytometry analysis.

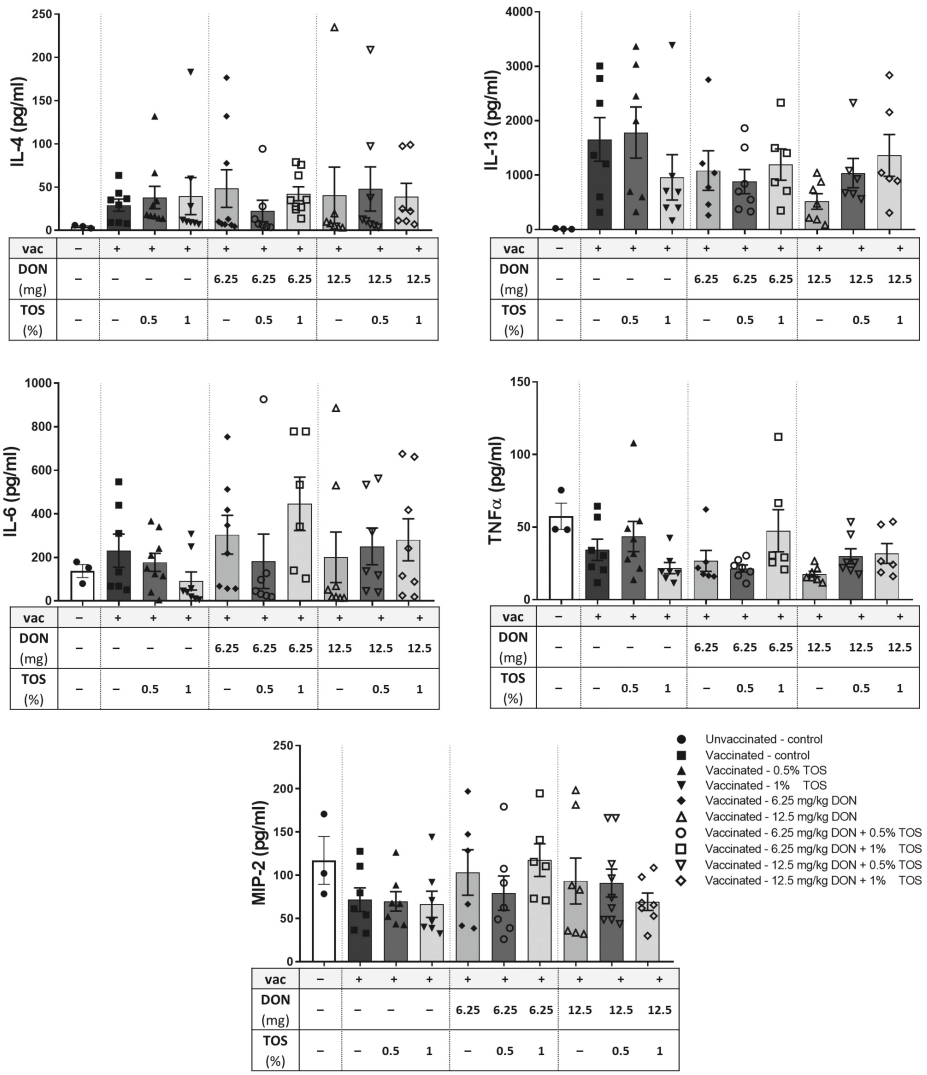
Marker	Label	Catalog number	Antibody ID	Dilution
CD4	BV510	100553	AB_2561388	1:160
CD69	PE-Cy7	25-0691	AB_469637	1:640
T1/ST2	FITC	101001F	AB_947549	1:200
CXCR3	PE	12-1831	AB_1210734	1:100
Tbet	Alexa Fluor647	644803	AB_1595573	1:1600
Gata3	PerCP-eFluor710	46-9966	AB_10804487	1:1000
CD25	PerCP-Cy5.5	45-0251	AB_914324	1:1200
CD196 (CCR6)	PE	129804	AB_1279137	1:640
FoxP3	FITC	11-5773	AB_465243	1:100
RorgT	Alexa Fluor647	562682	AB_2687546	1:400
AHR	PE-Cy7	25-5925	AB_2573501	1:200
CD3	Percp-cy5.5	45-0031	AB_1107000	1:100
CD19	APC	17-0193	AB_1659676	1:75
B220	FITC	11-0452	AB_465054	1:75
CD27	PE	12-0271	AB_465614	1:75

Supplementary table 2. List of relevant comparisons implemented in statistical analysis

Sham-treated group	vs.	Control (vaccinated)
Control (vaccinated)	vs.	0.5% TOS (wt/wt)
		1% TOS (wt/wt)
		DON (6.25 mg/kg of diet)
		DON (12.5 mg/kg of diet)
DON (6.25 mg/kg of diet)	vs.	DON (6.25 mg/kg) + 0.5% TOS
		DON (6.25 mg/kg) + 1% TOS
DON (12.5 mg/kg of diet)	vs.	DON (12.5 mg/kg) +0.5% TOS
		DON (12.5 mg/kg) + 1% TOS



Supplementary figure 3. Relative mRNA expression of tight junction proteins occludin (OCLD) and claudin-3 (CLDN-3) in ileum samples collected at day 31. Data are presented as mean ± SEM.



Supplementary figure 2. Cytokines production of co-cultured splenocytes with influenza-loaded bone marrow-derived DCs. Interleukin (IL)-4, IL-6, IL-13, tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-2 concentrations in culture supernatant.

Chapter 3

Exposing stimulated dendritic cells to deoxynivalenol induces Th2-polarizing immune responses, and inhibits B cell differentiation into IgM-producing plasma cells

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This chapter is submitted for publication.

Abstract

Deoxynivalenol (DON), a mycotoxin highly prevalent in cereal-based products, is known to induce immunotoxicity. Here, we demonstrate that exposing lipopolysaccharide (LPS)-stimulated human monocyte-derived dendritic cells (DCs) to DON inhibits their activation and cytokine production and modulates mRNA expression of Toll-like receptor-4 (TLR4)-signaling proteins. Coculturing DON-exposed DCs with allogeneic naïve $CD4^+$ T cells, decreased T cell activation and induced Th2-skewed differentiation along with an elevated IL-13 and reduced IFN-y production. Coculturing LPS-stimulated DCs with naïve B cells and T cells promoted B-cell differentiation to IgM-producing plasma cells, which was inhibited by exposing DCs to DON. Moreover, a reduced antigen-specific Th1-associated cytokine production was observed after coculturing splenocytes collected from Influvac-vaccinated mice with DON-exposed bone marrow-derived murine DCs loaded with Influvac. Exposure to DON thus appears to negatively affect DC-dependent cellular and humoral responses, possibly by interfering with the TLR4-signaling pathway in DCs, which as a consequence may influence host-pathogen interactions and trigger immune-related disorders.

Introduction

Mycotoxins are naturally produced fungal metabolites contaminating a wide range of human foods, especially cereal and grain-based products [1]. Mycotoxins generally have high thermal stability and are therefore difficult to eradicate during food processing [2]. Deoxynivalenol (DON), a trichothecene mycotoxin produced by Fusarium fungi species, is one of the most prevalent mycotoxins found in human food [3]. According to the BIOMIN World Mycotoxin Survey in 2020 [4], DON was detected in 65% of crops collected from 79 countries all over the world. As human exposure is thus virtually inevitable, it is essential to explore the toxicity of DON in humans [5]. DON is known to induce gastrointestinal, neurological, and reproductive toxicity; however, the immune system is considered to be the most sensitive to DON-induced toxicity [6]. Direct exposure to low concentrations of DON alters LPS-induced cytokine secretion, chemotaxis, and phagocytosis capacities in porcine polymorphonuclear cells [7], and reduces T cell proliferation and activation in peripheral blood mononuclear cells (PBMC) and modulates cytokine production capacity [8-10]. *In vitro* exposure of porcine monocyte-derived dendritic cells (DCs) to DON results in morphological changes and a reduction in DC maturation markers and endocytic activity [11]. Similarly, DON induced down-regulation of surface expression of major histocompatibility complex (MHC) class II and CD11c in murine bone marrow-derived DCs (BMDC) [12], and inhibited the LPS-induced up-regulation of maturation markers and secretion of maturation-associated cytokines such as Interleukin (IL)-10 and IL-12 in human DC [13].

A previous *in vivo* study showed that DON facilitates allergic sensitization to whey proteins in mice and enhanced initiation of Th2 responses [14]. Additionally it was demonstrated that oral administration of DON-contaminated diets reduces the immune responses to vaccination against Influenza virus, mainly by inhibiting Th1-mediated immunity and decreasing $Tbet^{+}$ Th1 cell populations in vaccinated mice [15]. These findings indicate that DON can interfere in adaptive immune responses, which warrants further investigation into how DON affects the early stages of acquired immunity. Herein, DC play a key role as they are responsible for antigen presentation and modulation of adaptive immune responses, such as T-cell and B-cell priming [16, 17]. Depending on the signals provided by DCs, T cells differentiate into a specific subset [18]. DC signalling is also prerequisite for T cell-dependent activation of naïve B cells and initiation and regulation of immunoglobulin synthesis [19, 20].

Although several studies have demonstrated toxic effects of DON on different immune cells, very little is known about the effect of DON on interactions between immune cells. In the present study, an *in vitro* allogeneic coculture model was used to study the interplay between human monocyte-derived DCs, T cells and B cells. This model proved to be very suitable to investigate the effect of DON exposure on DC maturation and activation, as well as naïve T cell and B cell activation and differentiation. Furthermore,

to evaluate the consequences of exposing DCs to DON on vaccination responsiveness, Influenza vaccine antigen-loaded murine BMDCs were used for *ex vivo* re-stimulation of splenocytes collected from vaccinated mice. Results in the present study demonstrate that exposing LPS-stimulated DCs to DON exposure induces Th2-skewing effects, and leads to reduced B cell differentiation to IgM-producing plasma cells. In the murine model, DON-exposed DCs induced diminished vaccine-specific Th1-associated cytokine production by splenocytes of vaccinated mice. Understanding the impact of DON on communication between immune cells can provide further insight in the immunotoxicity of DON, which may contribute to our knowledge on the role of DON in the human exposome.

Methods and materials

Isolation of peripheral blood mononuclear cells

PBMC were isolated from buffy coats from 9 healthy human donors (Sanquin, Amsterdam, Netherlands) by density-gradient separation. Cells were washed with phosphate-buffered saline (PBS; Lonza, Basel, Switzerland) supplemented with 2% fetal calf serum (FCS) and remaining erythrocytes were lysed by addition of red blood cell lysis buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg Na_2EDTA in 500 mL demi water, sterile filtered, pH = 7.4). PBMCs were resuspended in culture medium (RPMI 1640, Sigma Aldrich, St. Louis, USA) supplemented with 10% FCS, 1% penicillin (100 U/mL) and streptomycin (100 ug/mL).

Monocyte-derived dendritic cells

$CD14^+CD16^-$ monocytes were isolated from PBMCs by negative magnetic sorting, using a MACS Classical Monocyte Isolation kit (Miltenyi Biotec, Bergisch Gladback, Germany) according to the manufacturer's guidelines. DCs were generated by culturing isolated monocytes in RPMI medium, supplemented with 10% FCS, 1% penicillin (100 U/mL) and streptomycin (100 ug/mL), human recombinant GM-CSF (75 ng/mL, Prospec, Rehovot, Israel) and interleukin (IL)-4 (100 ng/mL, Prospec) to ensure differentiation of monocytes to immature monocyte-derived DCs. The cells were cultured for 6 days at 5% CO_2 at 37 °C. Medium was refreshed once on day 3.

Stimulation of dendritic cells in vitro

DCs were stimulated with LPS (100 ng/mL, InvivoGen, Toulouse, France) in the presence or absence of deoxynivalenol (DON, D0156-1MG, Sigma Aldrich) for 24 h. Preliminary studies showed a cytotoxic effect when DCs were incubated with concentrations of ≥8 µM DON; hence, lower concentrations (1, 2 and 4 µM DON) were used in this study. These concentrations are selected based on previous *in vitro* experiments, showing no significant reduction in cell viability with concentrations below 8 µM [11, 12, 21]. Non-stimulated DCs (without DON or LPS) served as negative controls. After culturing

for 24 h with DON, LPS or DON+LPS, DCs were washed with culture medium, in order to be used in coculture model.

T cell and B cell isolation and coculture with stimulated dendritic cells

Naïve $CD4^+CD45RA^+$ T cells and naïve B cells were isolated from PBMCs by negative magnetic sorting, using MACS Naïve $CD4^+$ T Cell Isolation Kit II and Naïve B Cell Isolation Kit II (Miltenyi Biotec), respectively, according to the manufacturer's guidelines. The phenotype of isolated naïve lymphocytes cells before coculturing with DCs was confirmed by flowcytometry analysis (supplementary figure 1). The isolated cells were resuspended in IMDM medium (Gibco, Gaithersburg, USA) supplemented with 10% heat-inactivated FCS, filter-sterilized apo-transferrin (20 µg/mL), 2-mercaptoethanol (50 µM), 1% penicillin (100 U/mL) and streptomycin (100 µg/mL). Next, LPS-stimulated DCs were resuspended in IMDM culture medium and cocultured either with freshly isolated allogeneic naïve T cells at a 1:10 ratio, or with combination of naïve T cells and B cells at a 1:5:5 ratio (total cell concentration was 500000 cells/mL in both coculture systems), for 6 days.

Cell viability determination by MTT assay

Cell viability and mitochondrial activity of DCs were determined using Cytoselect™ MTT Cell Proliferation Assay (Cell Biolabs, Inc., USA). Stimulated DCs from 4 donors were incubated with MTT for 4 h at 37°C and 5% CO2. The yellow MTT solution was converted to dark blue MTT formazan by mitochondrial dehydrogenases in live cells. The blue crystals formed in live cells were solubilized with dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm. Viability was calculated as a percentage of the absorbance measured in untreated control cells for each donor.

Real time qPCR

DCs and T cells were suspended in RNA lysis buffer with β-mercaptoethanol and RNA isolation was performed following the instructions of Promega's SV total RNA Isolation kit (Promega Corporation, Madison, USA). Final concentrations of RNA were measured using NanoDrop (ND One/One^c Microvolume UV-Vis spectrophotometer, Thermo Fisher, Waltham, USA). Complementary DNA (cDNA) was synthesised from isolated RNA by iScript cDNA Synthesis Kit (Bio-rad Laboratories, Hercules, USA), using T100 Thermal Cycler (Bio-rad Laboratories). For real time quantitative PCR (qRT-PCR) reactions, iQSYBR Green Supermix (Bio-Rad Laboratories) was mixed with cDNA and specific primer sets. Amplifications were performed in CFX96 Real-Time systems (Bio-rad Laboratories) according to manufacturer's instructions. All tested primers were purchased from Bio-Rad Laboratories. For primer efficiency and specificity, cDNA samples were pooled, and a temperature gradient was performed between 55 °C and 65 °C to determine melting curves and optimal annealing temperatures for each primer set. Using *B-actin* as a reference gene, relative mRNA expression levels were calculated.

Flow cytometric analyses

Phenotypes of DCs after stimulation, and of T cells and B cells after coculture were analysed by flow cytometry, using selected antibodies (supplementary table 1). Cells were first stained with fixable viability dye with fluorochrome eFluor780 (eBioscience, San Diego, USA); blocking buffer (1% bovine serum albumin (Roche, Basel, Switzerland), 5% FCS and human FC block (BD Biosciences, Franklin Lakes, USA), in PBS) were used to prevent non-specific binding of the antibodies. For detection of intracellular markers, the cells were fixed and permeabilized with Foxp3 Staining Buffer Set (eBioscience) prior to staining, according to the manufacturer's protocol. Stained cells were measured by BD FACS Canto II flow cytometer. The obtained data were analysed further using FlowLogic software (V.8, Inivai Technologies, Mentone Victoria, Australia).

Enzyme-linked immunosorbent assays (ELISA)

Supernatants collected from DCs cultures after stimulation, DC-T cell cocultures and DC-T cell- B cell cocultures were used for determining the concentration of specific cytokines and immunoglobulins (Igs). Commercially available ELISA kits were used to detect and quantify cytokines by following manufacturer's instructions. Concentrations of IL-10 (U-CyTech biosciences, Utrecht, Netherlands), IL-12 (Invitrogen, Carlsbad, USA) and IL-6 (Invitrogen) were measured in DC supernatant, and concentrations of Interferon-γ (IFN- γ), IL-10, IL-13, IL-2, and IL-21(Invitrogen) were measured in DC-T cell coculture supernatant. Concentrations of total IgM and IgG (Invitrogen) were measured in DC-T cell- B cell coculture supernatant.

Animals

Female C57Bl/6JOlaHsd mice (6-7 weeks old) were purchased from Envigo (Horst, The Netherlands) and conventionally housed in groups of 3-4 mice with a light/dark cycle of 12 h/12 h (lights on from 7.00 am–7.00 pm) at a controlled relative humidity (50–55%) and temperature (21 ± 2°C) with access to food and tap water ad libitum. This strain of mice was chosen based on previous studies. Upon arrival at the animal facility of Utrecht University, the animals were randomly allocated to filter-topped makrolon cages (22 cm×16 cm×14 cm, floor area 350 cm^2, Technilab- BMI, Someren, the Netherlands) with wood-chip bedding (Technilab- BMI, Someren, the Netherlands); tissues (VWR, the Netherlands) plastic shelters were available as cage enrichment. The animals were acclimatized for 2 weeks upon arrival in the animal facility, before the start of the experiments, and received standard pelleted diets (AIN-93G, Ssniff Spezialdiäten, Soest, Germany) and routine care throughout the experiment. All procedures were performed during light cycle. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the Utrecht University, and all animal procedures related to the purpose of the research were approved under license of the national competent authority, securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Isolating and stimulating bone-marrow derived DCs (BMDC)
Bone marrow cells were isolated from femurs and tibias of 5 healthy 11-week-old C57BL/6JOlaHsd mice after killing them by cervical dislocation. Cells were pooled and cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and Eagles minimum essential medium (MEM) non-essential amino acids (all from Gibco Life Technologies) in the presence of 10 ng/mL GM-CSF (Prospec, The Netherlands) for 6 days to obtain immature BMDC [22]. Subsequently, immature BMDC were loaded with Influvac vaccine at a concentration of 0.9 µg/mL, in presence of 1, 2 and 4 µM DON, and incubated for 24 h at 37°C, 5% CO2 to obtain matured DCs. Immature BMDC treated with medium were used as negative control.

Vaccination protocol and ex vivo BMDC-splenocyte coculture
After 2 weeks of acclimatization, mice (n=7) were vaccinated using Influvac (Abbott Biologicals B.V., Weesp, The Netherlands) from season 2019/2020 as previously described [22]. The mice received the primary and booster vaccinations by subcutaneous injections of 120 µl of undiluted Influvac (containing hemagglutinin (HA) and neuraminidase antigens of three strains of influenza virus, in a dose equivalent to 30 µg/mL HA per strain, totaling 90 µg/mL HA) in a skin fold of the neck. The booster vaccination was given 21 days after the primary vaccination. Sham-treated mice (n=3, negative control) received injections of 120 µl of PBS instead of vaccine in order to demonstrate the specificity of vaccine-induced immune response.

In order to determine the efficacy of vaccination, delayed-type hypersensitivity (DTH) responses were measured (in a blinded manner) 9 days after booster vaccination by intradermal (i.d.) injection of 20 µl Influvac into the ear pinnae of the right ear, and 20 µL PBS into the ear pinnae of the left ear, under isoflurane-induced anesthesia. Ear thickness was measured in duplicate before antigen challenge and 24 h thereafter using a digital micrometer (Mitutoyo Digimatic 293561, Veenendaal, The Netherlands). The antigen specific DTH responses were calculated by subtracting the basal ear thickness from the value at 24 h after challenge, correcting for the ear swelling that occurred as a result of s.c. injection of PBS using the formula:

(right ear (thickness at 24h - thickness at 0h)) - (left ear (thickness at 24h - thickness at 0h) = DTH (µm)

The validity of vaccination in this study was confirmed by measuring DTH as shown in supplementary figure 2.

Ten days after the booster vaccination, animals were killed by cervical dislocation and spleen cells were isolated as described previously [23]. After removal of red blood cells by incubating cell suspensions in lysis buffer (8.3 g NH4Cl, 1 g KHC3O, and 37.2 mg

EDTA dissolved in 1 L demi water and filter sterilized), splenocytes were counted and resuspended in RPMI 1640 medium containing 10% fetal bovine serum and penicillin (100 U/mL)/streptomycin (100 µg/mL). Freshly isolated splenocytes from vaccinated mice were cocultured with fresh, Influvac-stimulated BMDCs (collected from healthy non-vaccinated mice) at a 10:1 ratio, in 96-well U-bottom culture plates for 5 days at 37°C, 5% CO2, with supplemented RPMI 1640 medium (Gibco).

Determination of ex vivo antigen-specific cytokine production

Cell culture supernatants were collected at day 5 and analyzed for the concentration of IL-2, IL-6, IL-10, IL-12p70, IL-13, IL-21, IL-27 and IFN-γ using a ProcartaPlex multiplex protein assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

Statistical analysis

Descriptive statistical analyses were performed by using Graphpad Prism V8.4.3 (Graphpad, San Diego, USA). Normally distributed data sets (based on D'Agostino-Pearson test) were analysed with one-way analysis of variance (one-way ANOVA) for selected comparisons followed by Bonferroni post-hoc test; for not normally distributed data a Kruskal-Wallis test was performed, followed by Dunn's multiple comparisons test. The comparisons between control (unstimulated cells) and all other treatment groups, as well as the comparisons between LPS-treated and LPS+DON-treated cells were included in all analyses. Apart from the mRNA expression levels, which were normalized to the control-treated cells, repeated measures analysis was performed to compare the means of the parameters analysed in the current study. Data are presented as mean ± SEM, and $p < 0.05$ was considered statistically significant.

Sample size calculation

The required sample size for both human and murine models was calculated using G*Power v3.1.9. The sample size for human DC-T cell -B cell coculture model was calculated as n=8 based on the estimated effect size of 0.76. The effect size was calculated using the preliminary data on the effect of DON exposure on expression of maturation markers on LPS-stimulated DCs. The sample size for murine model of MBDC-splenocyte coculture model was calculated as n=7, with the effect size of 0.96. The effect size was based on previous data on effect of feeding mice a DON-contaminated diet on cytokine production from re-stimulated splenocytes [15]. Sham-treated group in murine model was used for validating the vaccination efficiency, therefore a group size of n=3 was proven to be sufficient. The power was set on 0.9 and α was corrected for the number of relevant comparisons in each model.

Results

DON exposure inhibits DC activation and modulates cytokine secretion

Flowcytometric analysis of DCs revealed no significant effect on cell viability after stimulation with LPS and/or DON (supplementary figure 3a and b). DCs were selected by gating DC-SIGN$^+$ cells from live cells. HLA-DR and CD80 surface expression was used to identify mature and activated DCs. Expression of activation marker CD80 was evaluated in matured HLA-DR$^+$ DCs, thus CD80$^+$ cells are gated out of HLA-DR$^+$ cells (gating strategy in figure 1a; representative FACS plots are shown in figure 2a and b). LPS stimulation significantly increased the percentages of HLA-DR$^+$ and CD80$^+$ DCs compared to the negative control. DON exposure without LPS had no effect on the expression of HLA-DR or CD80 expressions (figure 2c and d). Exposure to 1 µM DON slightly increased CD80$^+$ DCs, though not significantly (p=0.09). DON+LPS exposure significantly decreased HLA-DR expression by all concentrations of DON (figure 2c), and CD80 expression by 2 and 4 µM DON (figure 2d), compared to LPS alone.

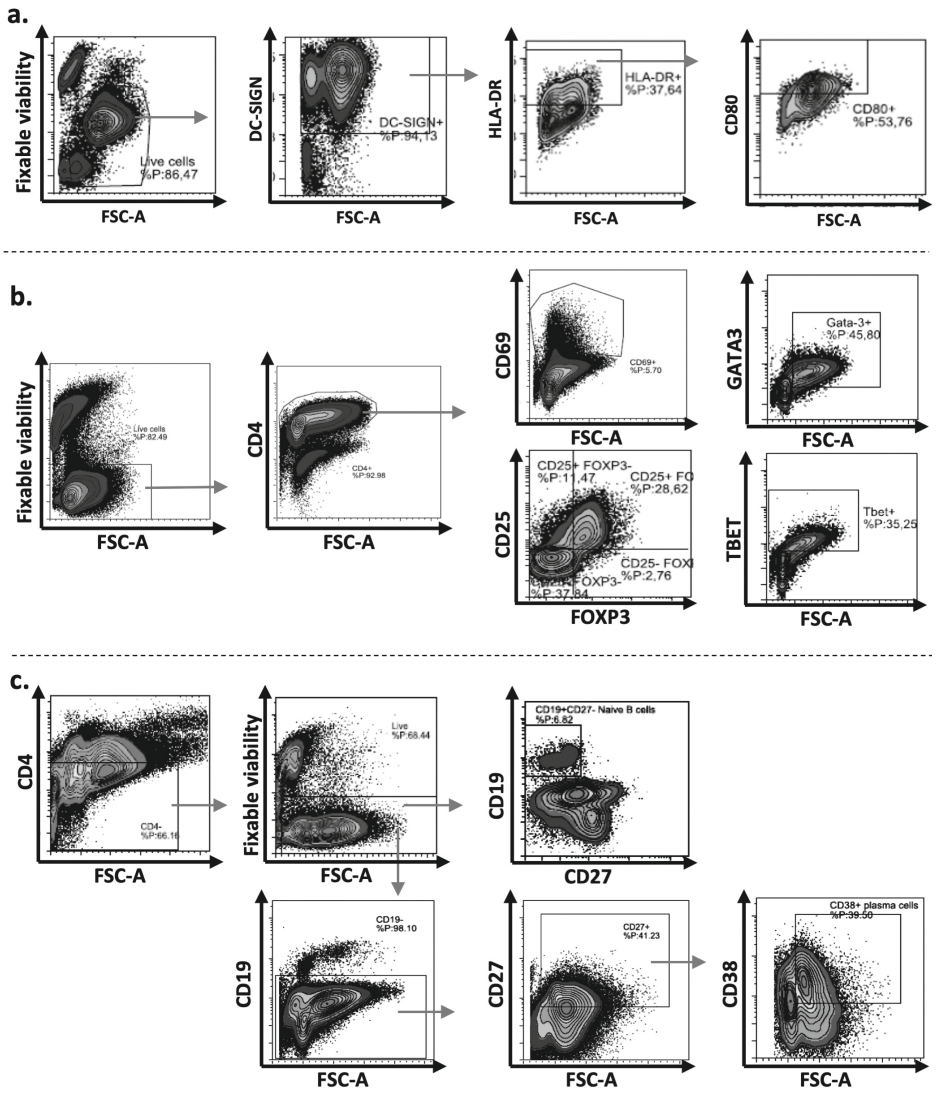

Figure 1. Gating strategy and representative plots for selecting: (**a**) matured HLA-DR$^+$ DCs out of live DC-SIGN$^+$ cells, and activated CD80$^+$ DCs out of matured DCs, (**b**) activated CD69$^+$ T helper (Th) cells, GATA3$^+$ Th2 cells, Tbet$^+$ Th1 cells, and FOxP3$^+$CD25$^+$ regulatory T cells out of live CD4$^+$ cells, (**c**) CD19$^+$CD27$^-$ naïve B cells and CD19$^{-(low)}$CD27$^+$CD38$^+$ plasma cells from live CD4– cells.

Concentrations of cytokines were measured in DC supernatants after 24 hours of stimulation. For all tested cytokines, a significant increase was observed between unstimulated and LPS-stimulated DCs (figure 2e, f and g), while no significant production of cytokines was observed by cells treated with DON alone. DON+LPS stimulation resulted in a significant reduction of IL-10 (all tested concentrations of DON, figure 2e)

and IL-12p70 secretion (2 and 4 µM DON, figure 2f), compared to LPS alone, while IL-6 secretion was reduced only by exposure to 4 µM DON (figure 2g).

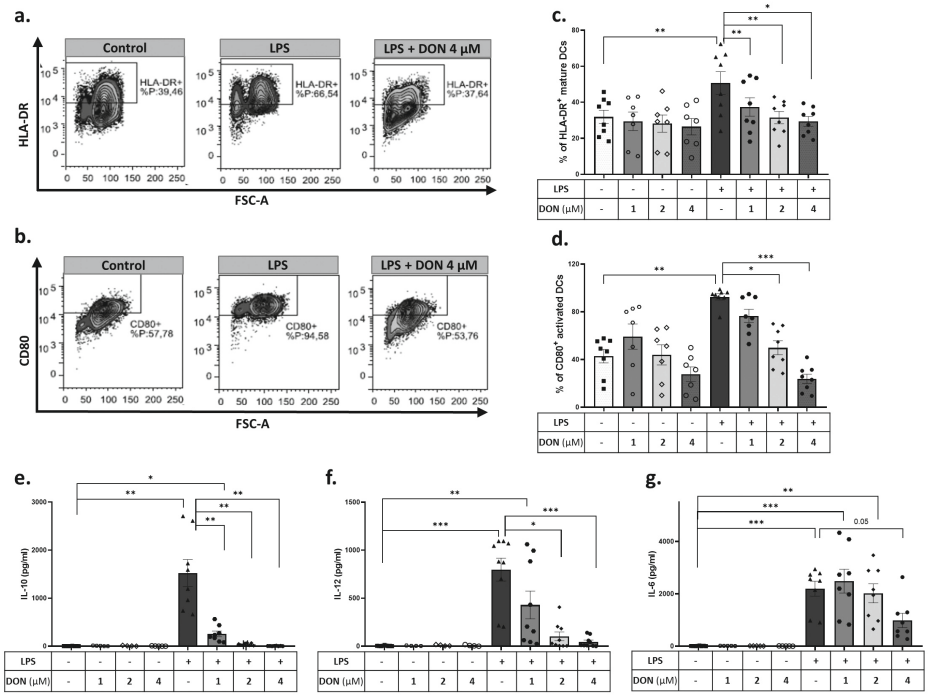

Figure 2. DON exposure modulates LPS-induced activation of DCs. Dendritic cells (DCs) were exposed to deoxynivalenol (DON; 1, 2 and 4 µM) and/or lipopolysaccharide (LPS; 100 ng/mL). The phenotype of DCs was studied after stimulation: (**a**) representative plots of HLA-DR$^+$ matured DCs gated form live DC-SIGN$^+$ cells; (**b**) representative plots of activated CD80$^+$ DCs gated from HLA-DR$^+$ DCs; (**c**) Percentage of mature HLA-DR$^+$ DCs, (**d**) Percentage of activated CD80$^+$ DCs. Concentrations of (**e**) interleukin (IL)-10, (**f**) IL-12, (**g**) IL-6 were measured in the supernatant. Data are presented as mean ± SEM of 8 independent donors (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

DON exposure modulates mRNA expression of DC activation and maturation markers

After stimulation, mRNA expression levels of activation and signal transducing proteins in DCs were assessed by qPCR. LPS stimulation had no effect on mRNA levels of *CD40*, myeloid differentiation factor 88 *(MyD88),* and the transcription factor interferon regulatory factor *(IRF)-4*, compared to unstimulated DCs (figures 3a-c). Stimulating DCs with LPS+DON (2 and 4 µM) significantly increased mRNA expression of these 3 markers, compared to LPS alone (figures 3a-c). On the other hand, compared to the unstimulated DCs, LPS-stimulation significantly reduced mRNA expression of Toll-like receptor 4 *(TLR4)* and integrin-alpha M *(ITGAM/CD11b*) (figures 3d and e), which are involved in LPS-induced signaling [24]. LPS+DON stimulation (4 µM) significantly increased the

mRNA expression of *TLR4,* compared to LPS alone ($p < 0.01$, figure 3d). Similarly, higher concentration of DON increased *ITGAM/CD11b* expression, though the effect is not statistically significant ($p = 0.07$, figure 3e). The mRNA expression of aryl hydrocarbon receptor (*AHR*), an immunoregulatory receptor responding to environmental factors [25], was not significantly changed by LPS stimulation, but additional exposure to 4 µM DON induced a significant increase in mRNA expression of *AHR* compared to control and LPS alone (figure 3f).

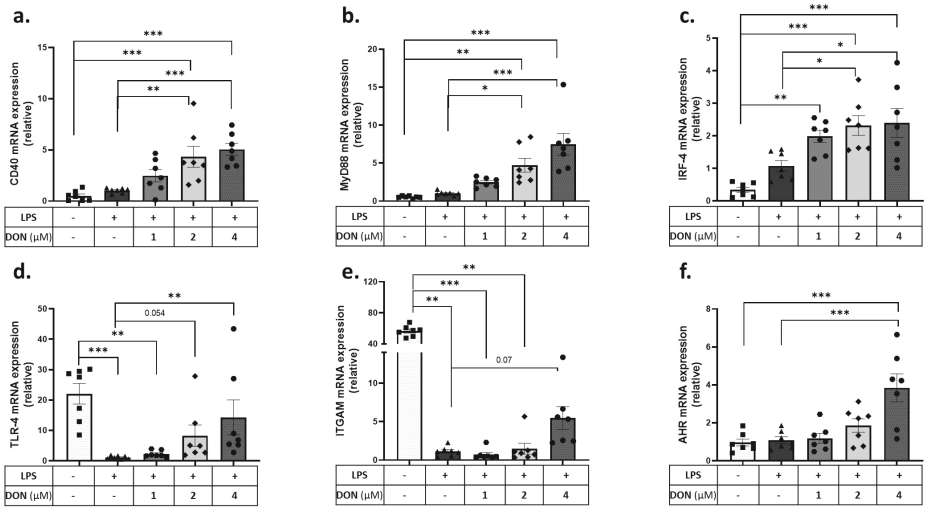

Figure 3. DON exposure in DCs modulate mRNA expression of DC differentiation and activation markers. Dendritic cells (DCs) were exposed to deoxynivalenol (DON; 1, 2 and 4 µM) and/or lipopolysaccharide (LPS; 100 ng/mL). Relative mRNA expression of (**a**) CD40, (**b**) myeloid differentiation factor 88 (MyD88), (**c**) interferon regulatory factor (IRF)-4, (**d**) toll-like receptor (TLR)-4, (**e**) integrin-alpha M (ITGAM/CD11b), and (**f**) aryl hydrocarbon receptor (AHR) were measure in DCs. Data are presented as mean ± SEM of 8 independent donors (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

DON-exposed DCs induce Th2-skewing effects and reduce T cell activation

After coculturing $CD4^+CD45Ra^+$ naïve T cells with DCs, the activation status of T cells and differentiation towards Th1, Th2 or Treg were studied by flow cytometry (gating strategy in figure 1b, representative FACS plots in figure 4a, c, e and g). No difference in T cell viability and total $CD4^+$ T cells were observed between different groups (supplementary figure 3c and d). LPS-stimulated DCs induced a significantly higher percentage of activated $CD69^+CD4^+$ T cells compared to unstimulated DCs. Stimulating DCs with LPS+DON (2 and 4 µM), resulted in significantly lower expression of CD69 on T cells, compared to LPS alone (figure 4b). Compared to unstimulated DCs, LPS-stimulated DCs induced no significant effect on the frequency of Tbet expressing T cells or $FOXP3^+CD25^+$ regulatory T cells (figure 4f and h), but percentages of $GATA3^+$

Th2 cells were lower (figure 4c). LPS+DON stimulation of DCs significantly increased GATA3 expression on T cells compared to LPS alone (figure 4c), while no differences were observed in percentages of $Tbet^+$ Th1 cells (figure 4f) and $FOXP3^+CD25^+$ Treg cells (figure 4h).

DON-exposed DCs modulate the secretion of cytokines in DC- T cell coculture

The supernatants of DC-T cell cocultures were used to determine the concentrations of T cell-associated cytokines. LPS-stimulated DCs did not affect IL-13 production, when compared to unstimulated DCs (figure 5a). However, additional exposure of DCs to DON (2 and 4 µM) significantly increased IL-13 production, (figure 5a). In contrast, a significant increase in the production of IFN-γ was induced by LPS-stimulated DCs, compared to unstimulated DCs (figure 5b). LPS stimulation of DCs together with 1 µM DON further increased the IFN-γ production, whereas exposure to 4 µM DON led to a significant reduction of IFN-γ secretion, compared to LPS alone (figure 5b). LPS-stimulation of DCs did not significantly affect IL-2 levels, while LPS+DON stimulation of DCs induced significantly higher IL-2 production by cocultured cells, compared to control and LPS-stimulated DCs (figure 5c). Analysis of IL-10 and IL-21 concentrations showed no significant differences between groups (supplementary figure 4a and b).

DON-exposed DCs modulate mRNA expression of activation and differentiation markers in T cells

After coculturing DCs with naïve T cells, mRNA was isolated from the cocultured cells, and the expression levels of different T cell activation and differentiation markers were assessed by qPCR. LPS-stimulated DCs did not affect mRNA expression of *GATA3* and *IL-2* in T cells (figure 5d and e), but upregulated *Tbet* mRNA levels (figure 5f), compared to unstimulated DCs. LPS+DON stimulation of DCs prior to coculture, significantly increased mRNA expression of *GATA3* with the concentrations of 4 µM, and decreased mRNA expression of *Tbet* compared to LPS alone (figures 5d and f). DON+LPS also induced significant increase in *IL-2* mRNA expression compared to cells cocultured with unstimulated DCs (figure 5e). No significant differences were observed in mRNA levels of *FOXP3*, *CD28*, *STAT1*, *STAT4* and *STAT6* (supplementary figure 4).

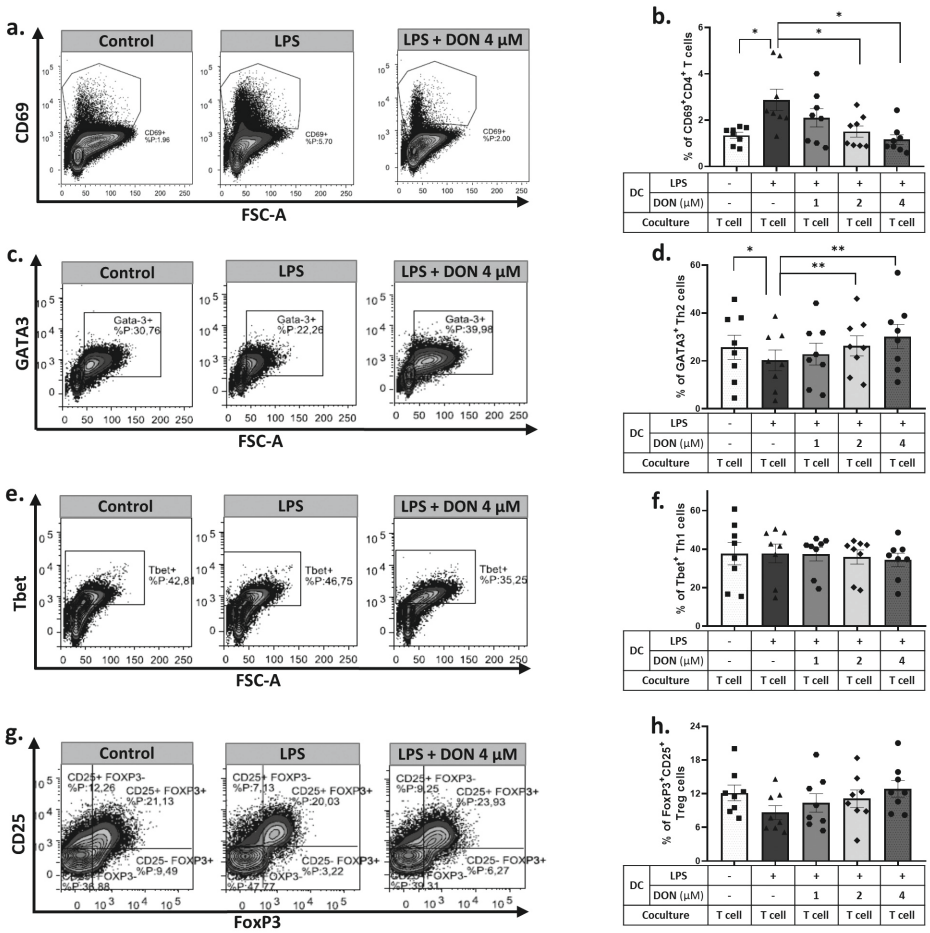

Figure 4. Exposure to DON modulates the capacity of DCs to activate and differentiate naïve T cells. Dendritic cells (DCs) were stimulated with lipopolysaccharide (LPS; 100 ng/mL) and deoxynivalenol (DON; 1, 2, and 4 µM), and were cocultured with allogeneic naïve $CD4^+$ T cells. The phenotypic changes of the $CD4^+$ T cells was assessed by flow cytometry. (**a**) Representative FACS plots of $CD69^+$ activated T cells gated from live $CD4^+$ T cells; (**b**) percentages of $CD69^+$ T cells; (**c**) representative plots of $GATA3^+$ Th2 cells gated from live $CD4^+$ T cells; (**d**) percentages of $GATA3^+$ Th2 cells; (**e**) representative plots of $Tbet^+$ Th1 cells gated from live $CD4^+$ T cells; (**f**) percentages of $Tbet^+$ Th1 cells; (**g**) representative plots of $CD25^+FOXP3^+$ regulatory T cells gated from live $CD4^+$ T cells; (**h**) percentages of $CD25^+FOXP3^+$ T cells. Data are presented as mean ± SEM of 8 independent donors. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

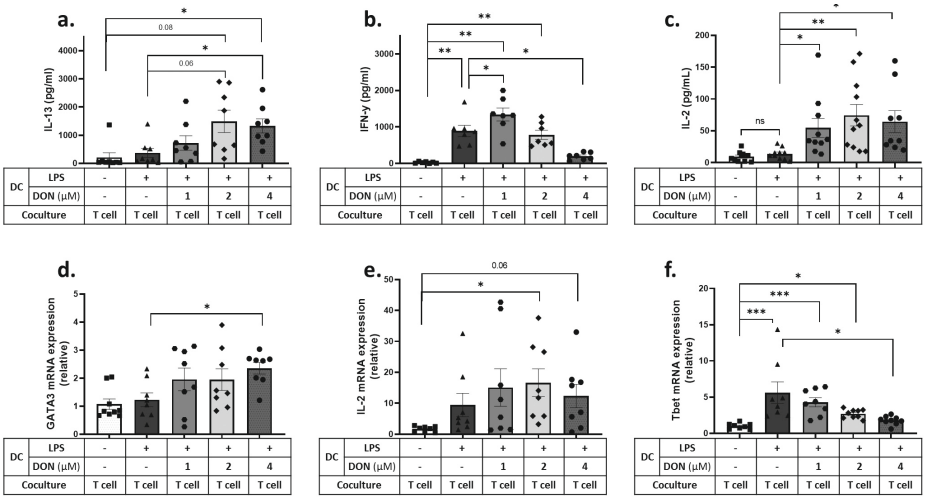

Figure 5. DON exposure in DCs modulates cytokine secretion in DC-T cell co-culture and mRNA expression of T cell markers. Dendritic cells (DCs) were stimulated with lipopolysaccharide (LPS; 100 ng/mL) and deoxynivalenol (DON; 1, 2, and 4 μM), and were cocultured with allogeneic naïve $CD4^+$ T cells. The concentrations of (**a**) interleukin (IL)- 13, (**b**) interferon (IFN)-γ, (**c**) IL-2 were measured in the supernatant. Relative mRNA expression of (**d**) GATA3, (**e**) IL-2, (**f**) Tbet were measured in cocultured cells. Data are presented as mean ± SEM of of 8 independent donors. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Triple coculture of DON-exposed DCs with T cells and B cells leads to reduction in B cell activation and IgM production

Activation and differentiation of B cells cocultured with DCs and T cells were analyzed with flowcytometry (gating strategy in figure 1c). $CD4^-CD19^+CD27^-$ cells were gated as naïve B cells and $CD4^-CD19^{-/low}CD27^+CD38^+$ cells were gated as plasma cells (figure 6a). Coculturing B cells and T cells with LPS-stimulated DCs with or without DON had no significant effect on B cell viability (supplementary figure 3e), or the percentage of naive B cells (figure 6b). Triple coculture with LPS-stimulated DCs increased percentage of plasma cells compared to unstimulated DCs, while LPS+DON stimulation of DCs inhibited the differentiation to plasma cells (figure 6c). Allogeneic coculture of naïve B cells with DCs without T cells, or naïve B cells with T cells without DCs led to significant reduction in cell viability and did not induce any plasma cell differentiation in B cells (supplementary figure 3f and g).

The supernatant of cocultured DCs-T cells-B cells was used to determine concentrations of total IgM and IgG. LPS-stimulated DCs induced significant production of IgM from B cells, compared to non-stimulated DCs, while addition of 2 and 4 μM DON to stimulated DCs significantly diminished IgM production by B cells (figure 6d). Total IgG production by B cells was increased by LPS-stimulated DCs, compared to unstimulated DCs (figure

6e). LPS+DON stimulation of DCs did not significantly affect total IgG production by B cells (figure 6e).

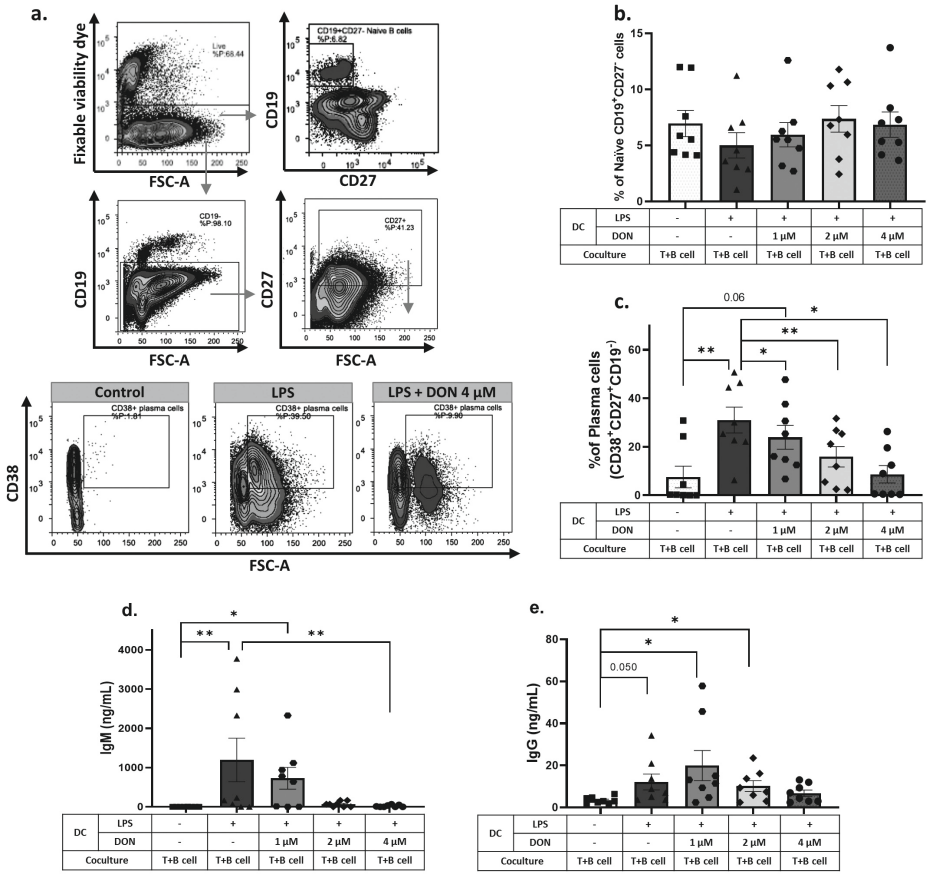

Figure 6. DON exposure in DCs inhibits B cell activation and immunoglobulin secretion. Dendritic cells (DCs) were stimulated with lipopolysaccharide (LPS; 100 ng/mL) and deoxynivalenol (DON; 1, 2, and 4 µM), and were cocultured with allogeneic naïve $CD4^+$ T cells and naïve B cells. The activation status of the B cells was assessed by flow cytometry. (**a**) gating strategy for selecting $CD19^+CD27^-$ naïve B cells and $CD19^{-(low)}CD27^+CD38^+$ plasma cells from live $CD4^-$ cells; (**b**) percentage of naïve B cells; (**c**) percentages of $CD4^-CD19^{-(low)}CD27^+CD38^+$ plasma cells. The concentrations of (**d**) total immunoglobulin (Ig)-M, and (**e**) total IgG were measured in the supernatant. Data are presented as mean ± SEM of 8 independent donors. (* $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$).

DON exposure in influenza-loaded BMDCs modulated cytokines production from splenocytes of vaccinated mice.

The effect of DON exposure on the capacity of BMDCs to stimulate cytokine production in immune competent cells was studied by *ex vivo* coculturing fresh splenocytes collected from vaccinated mice with antigen-loaded BMDCs. Compared to unstimulated BMDCs, concentrations of IL-10, IL-6, IFN-γ, IL-13 and IL-27 produced by splenocytes were significantly increased in by Influvac-stimulated BMDCs, while IL-2 production was significantly decreased (figure 7). The concentrations of IL-12p70, IL-33 and IL-21 in all groups were below the detection limit of the assay kit used in this experiment (data not shown). Addition of DON to Influvac-stimulated BMDCs caused a significant reduction in IL-6, IFN-γ and IL-27 production from splenocytes (figures 7c, d and g), while it had no significant effect on IL-10 and IL-13 concentrations (figures 7a and f), compared to Influvac-stimulated BMDCs. BMDC exposure to 4 µM DON led to a significant increase in IL-2 production compared to Influvac-stimulated BMDCs (figure 7b).

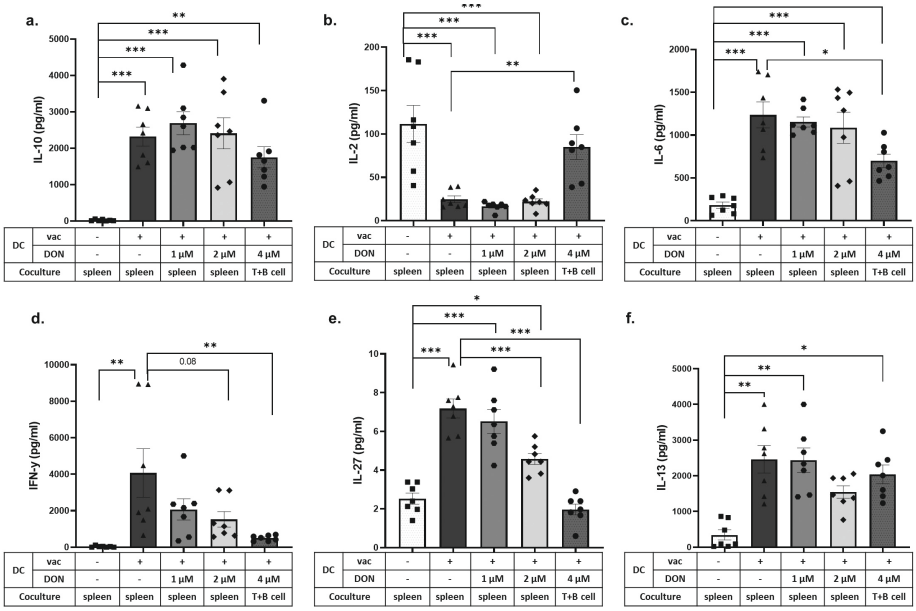

Figure 7. DON exposure in influenza-loaded BMDCs modulated cytokines production from splenocytes of vaccinated mice. Murine bone marrow-derived DCs (BMDCs) were stimulated with Influvac (0.9 µg/mL) and deoxynivalenol (DON; 1, 2, 4 µM), and were cocultured with splenocytes isolated from vaccinated mice for 6 days. Concentrations of (**a**) interleukin (IL)-10, (**b**) IL-2, (**c**) IL-6, (**d**) interferon (IFN)-γ, (**e**) IL-27, and (**f**) IL-13 were measured in cell supernatant. Data are presented as mean ± SEM, n=7. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Discussion and conclusion

DON is known to be a potent immunotoxic food contaminant, yet very little is known about its effects on immune cell interactions. The present study focused on the effect of DON on the capacity of DCs to activate naïve T cell and B cells, as the early steps in providing potent immune responses. The obtained results revealed significant modifications in immune responses of stimulated DCs due to DON exposure, which consequently translated to the modification of T cell and B cell activation and differentiation. Considering the importance of the dynamics of immune activation on downstream, health-related outcomes such as host-pathogen interactions and the initiation of non-communicable disease, DON exposure may warrant further caution.

First the effects of DON exposure on DCs was analyused, as these antigen presenting cells have an indisputable role in T cell activation and are therefore key in initiating adaptive immune responses. In line with previous findings [11, 13, 26], DON inhibited the upregulation of DC maturation and activation markers HLA-DR and CD80. This inhibition translated into functional differences, as stimulating DCs with LPS+DON prior to coculturing with naïve T cells led to a reduction in T cell expression of the early activation marker CD69. CD69 is the first detectable marker after ligation of T-cell receptor, and acts as a costimulatory molecule for T-cell proliferation [27, 28], but has additionally been suggested to have a regulatory function in inflammation [29]. A DON-induced reduction in the capacity of DCs to activate T cells may thus impact the CD69-regulated T-cell differentiation and cytokine production.

In addition to their essential function in activating naïve T-cells, the cytokine secretion profile of activated DCs is one of the most crucial factors in T-cell differentiation. Exposing LPS-stimulated DCs to DON prevented LPS-induced IL-10 and IL-12p70 production, as has been demonstrated before in human DCs, murine BMDCs, and porcine DCs [11, 13, 26]. Compared to IL-12 and IL-10, a less prominent effect of DON on LPS-induced production of IL-6 was observed, as it was only decreased with the highest concentration of DON used in this study (4 µM). IL-12 production by DCs enhances Th1 differentiation by priming naïve $CD4^+$ T cells for high IFN-γ production [30], while IL-6 polarizes $CD4^+$ T cells to effector Th2 cells and inhibits Th1 differentiation [31, 32]. IL-10 is an immunosuppressive cytokine, and its production by LPS-stimulated DCs inhibits the IL-12 secretion [33], hence acting as a regulatory mediator.

To assess whether DON-induced changes in DC-cytokine expression can affect the T-cell differentiation, the phenotype of T cells after coculturing with DON exposed DCs was investigated. LPS stimulation capacitates DCs to induce Th1 differentiation and increase IFN-γ production [34]. Exposing DCs to LPS+DON prior to coculturing with naïve T cells did not have any effects on the percentages of Tbet+ Th1 cells, but it downregulated Tbet mRNA expression levels in T cells. Furthermore, an increase in the percentage of $GATA3^+$

Th2 cells was detected after coculture with DON+LPS stimulated DCs, accompanied by upregulation of GATA3 mRNA expression in T cells, and an elevated production of the Th2-associated cytokine IL-13. These results indicate that DON-exposed DCs could be regarded as Th2-biased, favoring Th2 differentiation. Changes in the cytokine profile of DON-exposed DCs may, in part, be responsible for the observed Th2-polarizing effect; Although a reduction in LPS-induced IL-10, IL-12 and IL-6 production was observed in DCs, the effect of DON on IL-6, which favors Th2 differentiation, was less significant compared to IL-12 and IL-10. Considering the role of DC cytokines in differentiating T cells, an imbalanced production of cytokines by DCs may explain the imbalanced T-cell differentiation. Interestingly, exposing DCs to the lowest concentration of DON (1 μM) induced a higher IFN-γ production by T cells, while higher concentrations of DON (2 and 4 μM) led to a significant reduction. Previous studies have reported diminished mRNA and protein expression of IFN-γ by porcine T-cells after direct exposure to 100-800 ng/mL DON [35, 36], however, an important distinction between the latter studies and the current one is that in our experiments, T cells were not directly exposed to DON. Thus, the observed dual, concentration-dependent effect of DON on IFN-γ production may be partially explained by the altered ratio of IL-12 to IL-10 produced by DCs; IL-12 is known to promote the production of IFN-γ and other Th1-drived cytokines by $Tbet^+$ T cells [37]. However, since the production of IL-12 from DCs is negatively regulated by IL-10, a deficiency in IL-10 signaling can boost IL-12 signaling, leading to an increased IFN-γ secretion by T cells [38]. In line with these findings, we showed that exposure of DCs to 1 μM DON significantly inhibited LPS-induced IL-10 production, while it had no significant effect on IL-12 levels, which may have shifted the balance towards IL-12, subsequently resulting in a higher IFN-γ production by T-cells cocultured with these DCs. However, we observed that higher concentrations of DON significantly decreased both IL-12 and IL-10 release from DCs, which coincided with a diminished IFN-γ production by T-cells. Altogether, these observations indicate that DON exposure of DCs favors T cell differentiation towards Th2, and this effect is more prominent for higher concentrations tested in this study. This is further supported by our finding that stimulating DCs with LPS+DON prior to coculturing increased mRNA expression and production of IL-2 by T cells. IL-2 is a potent T cell growth factor, can augment immunoglobulin production [39], and is an essential factor for the development of regulatory T cells [39, 40]. IL-2 also regulates Th2 differentiation [41], and treating mice with anti-IL-2 inhibits priming for Th2-mediated cytokine production [40, 41]. Thus, an increase in IL-2 production, such as what we observed in T cells cultured with DON-exposed DCs, could further support the Th2-biased properties of these DCs. Interestingly, although DON+LPS stimulation inhibited the production of IL-10 by DCs, no differences in the percentages of $FoxP3^+$ Treg cells or IL-10 production by T cells was observed after coculture. This indicates that mechanisms other than changes in regulatory T cell responses are involved in the induction of imbalanced Th1/Th2 differentiation and cytokine production in $CD4^+$ T cells cocultured with DON-exposed DCs.

To further assess which factors contribute to the observed differences in T-cell differentiation, mRNA expression levels of several proteins involved in DC signaling pathways were measured. LPS is known to stimulate cytokine production in DCs through TLR4 signaling in a MyD88-dependent manner [34], and CD11b (ITGAM) acts as an additional signaling partner in MyD88-dependent T cell activation, facilitating LPS binding to TLR4 [24]. We found that in the absence of DON, LPS-stimulation induced a significant reduction in mRNA expression of surface proteins TLR4 and CD11b in DCs, while it had no effect of mRNA expression of cytoplasmic adaptor protein MyD88, compared to non-stimulated DCs. Downregulation of TLR4 and CD11b mRNA in DCs could be a cellular response to strong LPS-induced stimulation of these surface receptors. However, stimulating DCs with LPS+DON increased mRNA expression of TLR4, CD11b and MyD88, indicating that DON may interfere with LPS-induced TLR4 signaling in DCs. Exposure to DON may inhibit MyD88-dependent TLR signaling [42]; this is thought to result from a modulation of the mRNA expression of NF-κB and MyD88, and inhibition of NF-κB-dependent activity induced by MyD88-dependent TLR agonists [42, 43]. MyD88 is also important in DC-induced activation and differentiation of T cells; in MyD88$^{-/-}$ DCs, the production of IL-12 after LPS stimulation is abolished [34], and these DCs support Th2-polarization [44]. Inhibition of TLR-4/MyD88-associated signaling pathways in DCs can induce Th2-skewing effects in LPS-stimulated DCs [45, 46]. Thus, the observed Th2-biased properties of DON-exposed DCs could be linked to impaired TLR4-signaling in these cells. IRF-4 and CD40 are also involved in the LPS-induced DC activation and signal transduction pathway [47]. In the current study, LPS stimulation alone had no effect on mRNA expression of IRF-4 and CD40 markers, while LPS+DON exposure caused substantial upregulation of both markers in DCs. This may further explain the apparent bias toward Th2-differentiation after coculturing these DCs with naïve T cells, as both IRF-4 and CD40 are important for the initiation of Th2 responses [48-52]. Moreover, TLR activation is shown to induce mRNA expression of IRF-4, which binds to MyD88 and participates in the negative-feedback regulation of TLR signaling [53]. Thus, DON-induced changes in IRF-4 mRNA expression may contribute to the observed reduction in DC activation.

Next to the effect on LPS-induced signaling pathways, a significant increase in AHR mRNA expression in DCs stimulated by LPS+DON was observed. AHR is a cytosolic receptor protein regulating immune responses to environmental and physiological stresses [25]. The increase in expression of AHR can be linked to the observed reduction in IL-12 production in DCs due to DON and diminished Th1-associated responses in T cells, as exposure to AHR-ligands is shown to diminish IL-12p70 production in DCs [54], and reduce the mRNA expression of Tbet and IFN-γ in naïve murine T cells [55]. Nevertheless, further investigations are required to confirm whether DON is acting as an AHR ligand in DCs.

To further explore the effect of DON exposure on the initiation of the adaptive immune response, the DC – T cell coculture model was extended to include naïve B cells as well. An induction of differentiation of naïve B cells to plasma cells and an increase in the production of IgM and IgG was found after coculture with naïve T cells and DCs that had been stimulated with LPS prior to coculturing. This effect was dependent on the presence of both the T cells and the DCs. Stimulated DCs and DC-activated Th cells are known to interact with B cells to trigger the differentiation into IgM-secreting plasma cells [51, 56]. Interestingly, LPS-induced antigen-independent activation of B cells was observed in the *in vitro* coculture model. LPS-induced DC-signaling [57], along with bystander T-cell signaling through CD40L and cytokine production could possibly be responsible for this observation [58].

Exposing DCs to LPS+DON prior to coculturing inhibited B-cell differentiation to plasma cells and IgM production. In line with these observations, it has previously been shown that direct exposure of stimulated peripheral blood lymphocytes to DON leads to a significant reduction in IgG and IgM secretion [36, 59]. The cytokine profile of DCs and T cells are important in the regulation of B cell differentiation [58, 60]. A possible explanation for the observed reduction in plasma cell differentiation and IgM production could be the reduced capacity of DON-exposed DCs to produce IL-12 and activate T cells. However, further investigation is required to fully elucidate the mechanism underlying the observed effects of DON on humoral immunity.

To further validate the biological relevance of the findings on DON-induced changes in immune activation, we examined whether DON would affect the capacity of murine BMDC loaded with influenza vaccine antigens to induce cytokine release in splenocytes derived from vaccinated mice. Murine splenocytes cocultured with DON-exposed BMDCs produced less Th1-associated IFN-γ and IL-27, while concentrations of Th2-associated cytokines IL-13 and IL-5, and regulatory cytokine IL-10 were not affected. Exposing vaccine-loaded BMDCs to 4 μM DON significantly reduced IL-6 and elevated IL-2 production from splenocytes. These observations are in line with the results of exposing human DCs to DON and further support the notion that DON exposure leads to reduced Th1-associated responses. In an earlier *in vivo* experiment in our group, mice fed with DON-contaminated diets showed significantly lower immune responses after vaccination against Influenza virus, demonstrated by lower vaccine-specific serum IgGs and decreased Th1-associated immune responses in the spleen [15]. These observations may, in part, be explained by the inhibitory effect of DON on Th1-polarizing capacity of DCs. Lower Th1-mediated responses due to DON exposure may subsequently hamper proper vaccination responses and might interfere with effective immunity against microbes and viruses.

In summary, an *in vitro* model was developed to study DC-T cell-B cell interactions in absence or presence of DON. DON exposure of DCs inhibited LPS-induced activation and cytokine production, and hence attenuated the capacity of DCs to activate naïve $CD4^+$ T cells. Coculturing T cells with DCs stimulated with LPS+DON induced Th2-biased differentiation of T cells, which may be linked to the impaired TLR4-mediated signaling in DCs. Moreover, DON exposure of DCs inhibited T cell-dependent B cell activation and IgM production. Altogether, these results show that exposure of antigen presenting cells to DON can affect adaptive cellular and humoral immunity, which may ultimately negatively affect host immune responses to pathogens and trigger the initiation of immunologic disorders, mainly by shifting Th1/Th2 balance. Although further investigation is required to explicate the exact mechanisms involved in DON-induced immunotoxicity, these findings clearly demonstrate the importance of studying the immunomodulatory potential of this mycotoxin which is so ubiquitously present in food products.

Author contributions

NST, AH: designed the research; AH, BVL, JG: supervised data interpretation; NST, IVL: conducted the experiments; NST, IVL: analyzed data; NST: wrote the paper; GF, AH, JG, BVL, SB: read and approved the final manuscript; All authors provided critical intellectual input for data interpretation.

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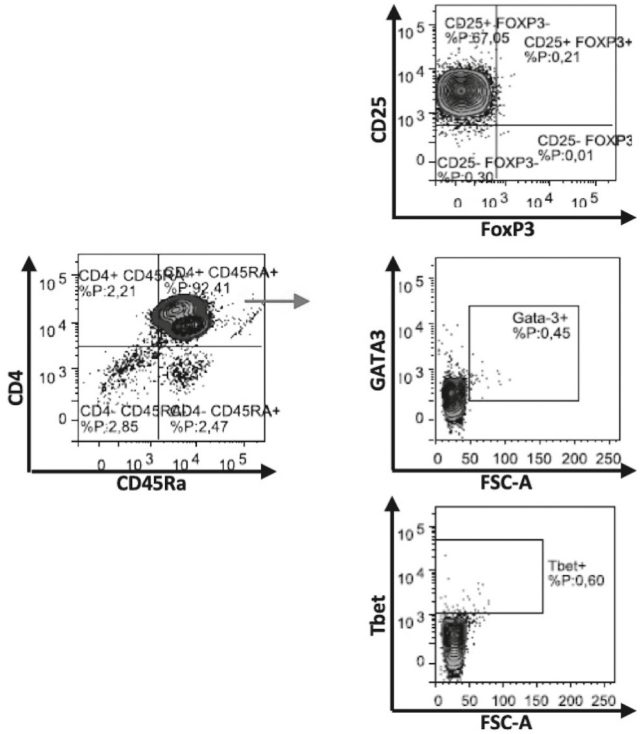
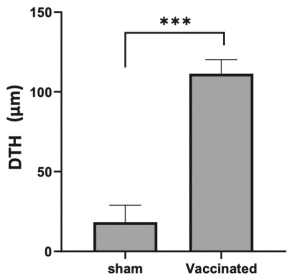
Supplementary content

Supplementary table 1 Antibody overview used for flow cytometry.

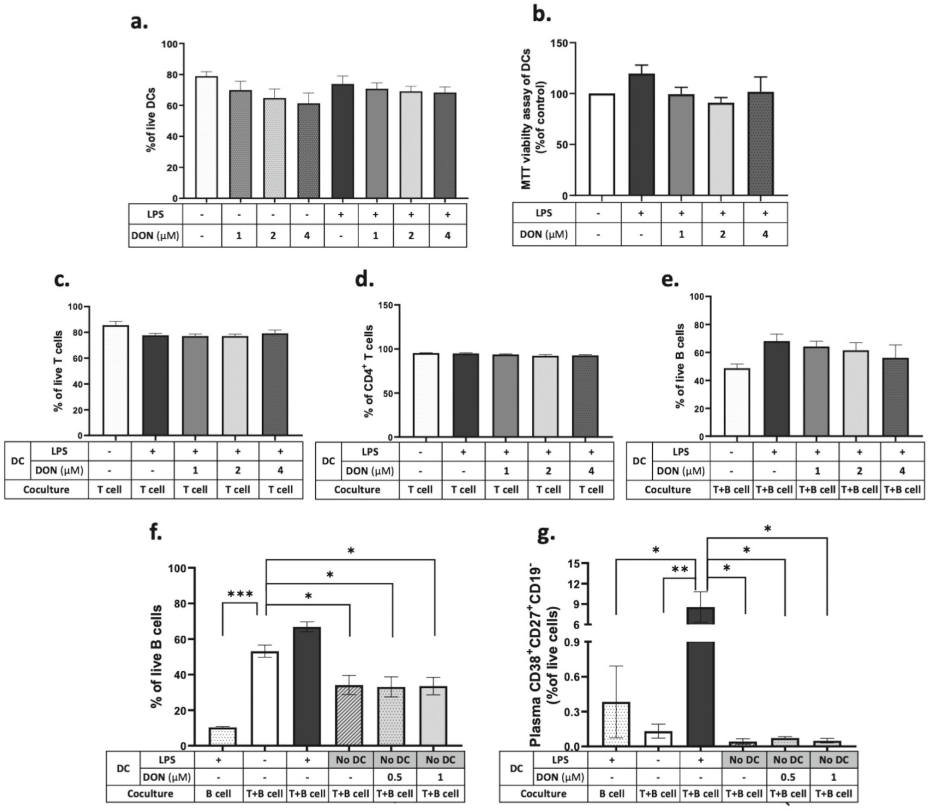
Antibody	Supplier	Identifier
DC Staining		
CD80 (B7-1) Monoclonal Antibody (2D10.4), FITC	eBioscience	AB_10853194
HLA-DR Monoclonal Antibody (L243), PE	eBioscience	AB_1272164
CD14 Monoclonal Antibody (61D3), eFluor 450	eBioscience	AB_1272120
CD209 (DC-SIGN) Monoclonal Antibody (eB-h209), APC	eBioscience	AB_11039758
T cell staining		
CD4 Monoclonal Antibody (RPA-T4), eFluor 506	eBioscience	AB_2637465
CD25 Monoclonal Antibody (BC96), Alexa Fluor 488	eBioscience	AB_2043828
CD69 Monoclonal Antibody (FN50), eFluor 450	eBioscience	AB_2574024
FOXP3 Monoclonal Antibody (PCH101), APC	eBioscience	AB_1603280
T-bet Monoclonal Antibody (eBio4B10 (4B10)), PerCP-Cyanine5.5	eBioscience	AB_953658
Gata-3 Monoclonal Antibody (TWAJ), PE	eBioscience	AB_1963600
B cell staining		
CD4 Monoclonal Antibody (RPA-T4), eFluor 506	eBioscience	AB_2637466
Pacific Blue™ anti-human CD19 (HIB19)	Biolegend	AB_493652
CD27 Monoclonal Antibody (O323), PerCP-eFluor 710	eBioscience	AB_1834392
CD38 Monoclonal Antibody (HIT2), APC	eBioscience	AB_10547344



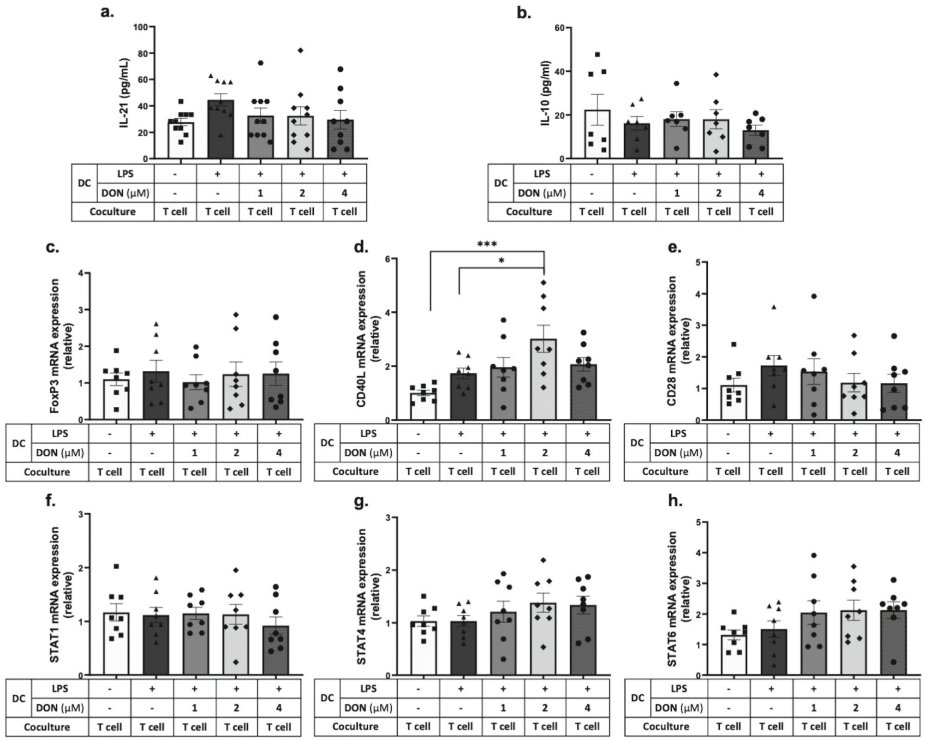
Supplementary fig. 1 Gating strategy and representative plots for selecting $CD4^+CD45Ra^+$ naïve T cells after isolation, and $CD25^+FoxP3^+$ Treg cell, $GATA3^+$ Th2 cells and $Tbet^+$ Th1 cells in isolated naïve T cells.



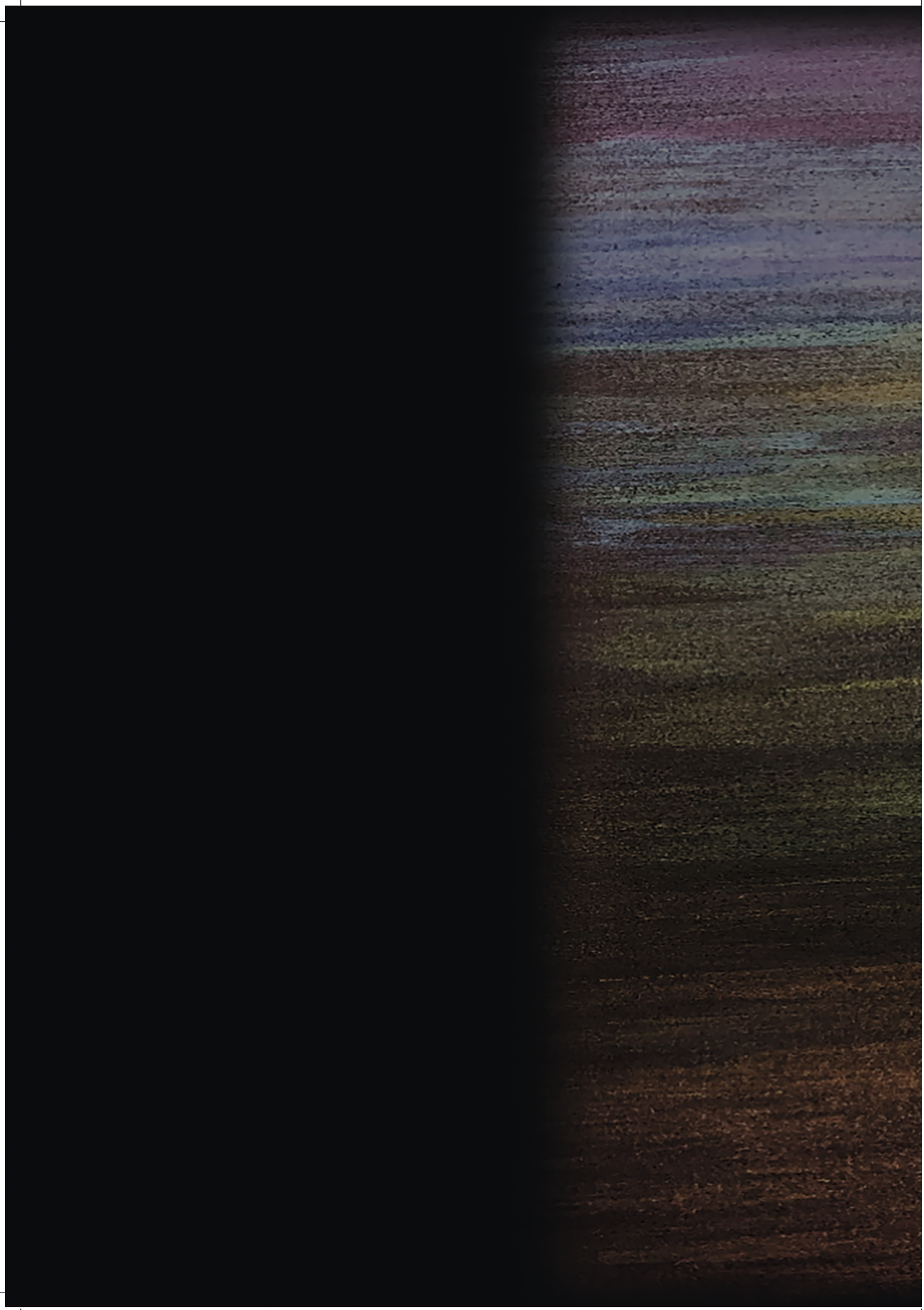
Supplementary fig. 2 Effect of vaccination with Influvac on vaccine-specific delayed-type hypersensitivity (DTH) responses. Data are presented as mean ± SEM of 3 mice in sham group and 7 mice in vaccination group (*** $p < 0.001$).



Supplementary fig. 3 Cell viability. DCs were stimulated with different concentrations of DON (1, 2, and 4 μM) in the presence or of LPS (100ng/ml) for 24 h, and were co-cultured with allogeneic naïve T cells or combination of naïve T cells and naïve B cells for 6 days. (**a**) percentages of viable DC measured by flowcytometry, and (**b**) results of MTT assay on DCs after stimulation, represented as percentage of control; (**c**) T cell viability, and (**d**) percentages of live $CD4^+$ T cells measured by flowcytometry after co-culture; (**e, f**) percentages of viable B cells, and (**g**) percentages of plasma cells measured by flowcytometry after co-culture Data are presented as mean ± SEM of 7-8 independent donors. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)



Supplementary fig. 4 Dendritic cell (DC) were stimulated with lipopolysaccharide (LPS; 100 ng/mL) and deoxynivalenol (DON; 1, 2, and 4 µM), and cocultured with allogeneic naïve $CD4^+$ T cells. Concentrations of (**a**) interleukin (IL)-21 and (**b**) IL-10, were measured in the cell supernatant. Relative mRNA expression of (c) FoxP3, (d) CD40 ligand (CD40L), (e) CD28, (f) Signal transducer and activator of transcription 1 (STAT1), (g) STAT4, and (h) STAT6 were measured in cocultured cells. Data are presented as mean ± SEM of 8 independent donors. (* $p < 0.05$, *** $p < 0.001$)



Part 2

Impact on Pregnancy

Chapter 4

Fusarium mycotoxins disrupt the barrier and induce IL-6 release in a human placental epithelium cell line

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Abstract

Deoxynivalenol, T-2 toxin, and zearalenone, major Fusarium mycotoxins, contaminate human food on a global level. Exposure to these mycotoxins during pregnancy can lead to abnormalities in neonatal development. Therefore, the aim of this study was to investigate the effects of Fusarium mycotoxins on human placental epithelial cells. As an in vitro model of placental barrier, BeWo cells were exposed to different concentrations of deoxynivalenol, zearalenone or T-2 toxin. Cytotoxicity, effects on barrier integrity, paracellular permeability along with mRNA and protein expression and localization of junctional proteins after exposure were evaluated. Induction of proinflammatory responses was determined by measuring cytokine production. Increasing mycotoxin concentrations affect BeWo cell viability, and T-2 toxin was more toxic compared to other mycotoxins. Deoxynivalenol and T-2 toxin caused significant barrier disruption, altered protein and mRNA expression of junctional proteins, and induced irregular cellular distribution. Although the effects of zearalenone on barrier integrity were less prominent, all tested mycotoxins were able to induce inflammation as measured by IL-6 release. Overall, Fusarium mycotoxins disrupt the barrier of BeWo cells by altering the expression and structure of junctional proteins and trigger proinflammatory responses. These changes in placental barrier may disturb the maternal–fetal interaction and adversely affect fetal development.

Introduction

Mycotoxins are naturally produced as secondary metabolites of fungi species and contaminate a wide range of foods, especially cereal and grain products [1]. Fusarium species are one of the most prevalent contaminants of cereal grains. Deoxynivalenol (DON), T-2 toxin and Zearalenone (ZEN) are the major Fusarium mycotoxins occurring in human food worldwide [2]. Despite all efforts and legislations to limit the fungal contamination, human exposure to mycotoxins cannot be completely prevented, as they are natural contaminants of agricultural products, and the invasion of toxigenic fungi species occurs regularly in food supplies at a global level [3]. Moreover, the masked or metabolized mycotoxins are of great concern in risk assessments because they are hardly detected by the conventional analytical methods and can transform to the parent mycotoxins after ingestion [4]. Due to the variation in mycotoxin content of human foods and seasonal differences in fungal infection, it is difficult to make an exact estimation of human exposure level. However, investigating the occurrence of Fusarium mycotoxins and their modified forms in cereal grains originating from different European countries shows a considerable level of contamination exceeding the maximum tolerable daily intake (TDI) in some cases [5]. As exposure is inevitable, it seems essential to explore the health effects of different mycotoxins in humans, especially high-risk groups, including fetus, neonates and infants [6].

Several studies have demonstrated that DON, ZEN and T-2 toxin can pass through the placenta [7-9] and adversely affect the development of the fetus during pregnancy. DON, ZEN and their major metabolites are detected in fetal and placental samples of pregnant sows and rats receiving contaminated diets during pregnancy [8, 10, 11]. DON exposure in pregnant mice can lead to increased resorption rate, structural and functional damages in placenta and fetal skeletal malformations [12, 13]. Prenatal exposure to ZEN, as a non-steroidal estrogenic mycotoxin, can cause long-term adverse effects on the reproductive system of the first-generation female offspring (F1), including abnormal and unfunctional ovarian follicles [14]. Studies have shown that ZEN can inhibit the mRNA expression of the estrogen receptor α (Esr1) and alter the expression of major ABC transporters in placentae collected from pregnant rats, and in BeWo cells *in vitro*, indicating that this mycotoxin can alter placental transportation, and consequently increase the risk of exposure to different xenobiotics and toxins in fetuses [14-16]. Studies have shown that T-2 toxin can also pass through the placenta and distribute to fetal tissues [17], which can induce fetal death, brain damage [18, 19], skeletal malformation [20], thymus atrophy [21], and suppression of humoral immunity [22].

During pregnancy, the placenta is the only link between the fetus and mother, mediating maternal-fetal transfer of nutrients and metabolic waste products, while also secreting hormones that maintain pregnancy [23]. The placenta is also important as a barrier

against pathogens and paracellular diffusion of chemicals and toxins [24]. The epithelial layer of the placenta consists of cytotrophoblasts and syncytiotrophoblasts, and controls maternal-fetal transfer [25]. Therefore, any disruption of the integrity of this layer can lead to imbalanced maternal-fetal transportation of nutrients and hazardous chemicals. In the placental epithelium, junctional proteins - including tight junctions (TJs) and adherent junctions (AJs) - are responsible for the preservation of this barrier. These proteins maintain the integrity and control paracellular transport of macromolecules across the placental epithelium [26-28]. Pathophysiological conditions that trigger the inflammatory responses in placenta can result in disassembly of junctional proteins at trophoblast cells and weaken the integrity, leading to detrimental consequences for the development of the fetus [29].

There are no reports on the effects of Fusarium mycotoxins on the placental barrier function. Therefore, in this study the effects of major Fusarium mycotoxins on the placental barrier function have been investigated by evaluating the expression and localization of junctional proteins. In addition, cell viability and inflammatory responses in placental epithelium were determined after mycotoxin exposure. To this end, BeWo cells were used as an *in vitro* model for placental epithelium [23]. This cell line is derived from human choriocarcinoma which retain the cell properties similar to the human trophoblasts, which are in direct contact with maternal blood supply [30]; therefore, it serves as a suitable *in vitro* model of the rate-limiting barrier to maternal–fetal exchange [24]. Untreated BeWo cells show the morphological and biochemical characterizations of undifferentiated cytotrophoblasts [31], and therefore, can be a representative of placental epithelium of the first trimester of pregnancy.

Materials and methods

Mycotoxins

Purified DON, ZEN and T-2 toxin (D0156, Z2125, T4887, Sigma-Aldrich, St Louis, MO, USA) were dissolved in absolute ethanol, methanol and chloroform, respectively, to prepare stock solutions according to the manufacturer's instructions, and were stored at −20°C. The stock concentrations of DON and ZEN were 32 mM and T-2 toxin was 4 mM Serial dilutions were prepared in cell culture medium prior to each experiment.

Different concentrations of DON (1, 2, 4, 8 and 16 µM), ZEN (1, 2, 4, 8 and 16 µM) and T-2 toxin (1, 2, 4, 8, 16 and 32 nM) were used in the assays described below. An incubation time of 24 h was used throughout the experiments related to the daily exposure to these regular food contaminants in an *in vivo* situation.

Cell culture

The human placenta choriocarcinoma (BeWo) cell line was obtained from the American Type Culture Collection (ATCC-CCL-98, Rockville, MD, USA; passages 11–45) and was

cultured in culture flasks (Falcon, VWR International, Strasbourg, France) in F-12K medium (Kaighn's modification of Ham's F-12 medium; Gibco, Thermo Fisher Scientific, Wilmington, DE, USA), supplemented with 10% fetal bovine serum (Perbio Science, Brebières, France), and 1% penicillin (100 U/ml) and /streptomycin (100 µg/ml) mixture. Cells were maintained in a humidified atmosphere of air containing 5% CO2 at 37 °C. Medium was refreshed three times per week and confluent cell cultures (7 ± 1 days) were passaged using 0.05% trypsin (Gibco, Thermo Fisher Scientific, Wilmington, DE, USA) and 0.54 mM ethylene diamine tetra-acetic acid (EDTA).

For mycotoxin exposure, BeWo cells were seeded in 96 or 24 well plates (Falcon, VWR International, Strasbourg, France) at cell densities of 10,000 and 30,000 cell per well, respectively. Once achieving 80% confluency (after 7 days), the cells were exposed to one of the abovementioned mycotoxins for 24 h.

Cell viability assay

Cytotoxic effects of different concentrations of DON (1, 2, 4, 8 and 16 µM), ZEN (1, 2, 4, 8 and 16 µM) and T-2 toxin (1, 2, 4, 8, 16 and 32 nM) on BeWo cells were determined by measuring LDH release from lysed and dead cells in cell culture medium using the CytoTox 96® Non-Radioactive CytoToxicity Assay Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Cytotoxicity (%) was calculated using the formula: Cytotoxicity (%) = (E/M) x 100% , where E is the experimental release of LDH, and M is the maximal release of LDH determined by incubating the cells with a standard lysis solution included in the assay kit. A wide range of mycotoxin concentrations were initially used to make a concentration-response assessment and determine the minimum concentrations of the mycotoxins, which can impose toxic effects on the placental epithelial barrier. These mycotoxin concentrations are also related to the concentrations used in other cell-based assays [32-34]. In subsequent experiments, mycotoxins were used at concentrations equal to or below the minimum concentration, which caused a significant cytotoxic effect and cell death.

TEER measurement and paracellular tracer flux assay

BeWo cells were seeded on high-pore-density polyethylene terephthalate membrane transwell inserts with 0.4 µm pores and surface area of 0.3 cm^2 (Falcon; BD Biosciences, Franklin Lakes, NJ, USA) placed in a 24-well plate at cell density of 30,000 cells per transwell insert. The integrity of the cellular monolayer was evaluated by measuring transepithelial electrical resistance (TEER) using a Millicell-ERS volt-ohm meter (Millipore, Temecula, CA, USA). After 7 days mean TEER values for untreated confluent cell monolayers were 60 ± 0.6 $\Omega \cdot cm^2$, as also observed in other BeWo cell cultures [24, 33, 35, 36]. The increasing concentrations of DON, T-2 toxin or ZEN were added to the apical side of the cells and TEER was measured 24 h after mycotoxin exposure. Thereafter, membrane-impermeable FITC-D (molecular mass of 4 kDa, 16 µg/ml; Sigma-Aldrich, St Louis, MO, USA) was added to the apical compartment for 3 h, and paracellular flux was

determined by measuring the fluorescence intensity in the basolateral compartment with a fluorometer (Fluoroskan Ascent FL; Thermo Labsystems; Waltham, MA, USA) set at excitation and emission wavelengths of 485 nm and 520 nm.

RNA extraction and quantitative qPCR

BeWo cells, seeded in 24-well plates as described above, were exposed to different mycotoxins for 24 h. Cells were harvested into 100µl RNA lysis buffer with β-mercaptoethanol (provided within RNA isolation kit) and total RNA was isolated using spin columns based on manufacturer's instructions (SV Total RNA Isolation System, Promega Corporation, Madison, WI, USA). Total RNA content of the samples was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The RNA purity of the samples was confirmed by calculating the 260/280 nm and 260/230 nm ratios, and the samples with the ratios between 1.8 to 2 were considered as high purity. Subsequently, the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to reverse-transcribe the RNA into cDNA, using the T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).

For qPCR, the reaction mixture was prepared by adding specific forward and reverse primers and iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) to the samples, and amplifications were performed according to manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Selected primers (Table 2) [37] were commercially manufactured (Biolegio, Nijmegen, Netherlands) and for specificity and efficiency confirmation, qPCR with dilution series of pooled cDNA at a temperature gradient of 55 °C to 65 °C was performed to analyze the melting curves and determine the optimum annealing temperature for each primer. The mRNA quantity was calculated relative to the expression of β-actin reference gene.

Western blot analysis

In order to quantify the protein expression of tight junctions in BeWo cells, western blot analysis was performed. The cells, seeded in 24-wells plates as described above, were exposed to different mycotoxins for 24 h. Cells were lysed and harvested with RIPA Lysis and Extraction Buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany). Total protein concentration was assessed by a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amount of protein from heat-denaturized samples were separated by electrophoresis (Criterion™ Gel, 4–20% Tris–HCl, Bio-Rad Laboratories, Hercules, CA, USA) and transferred by Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA) onto Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad Laboratories, Hercules, CA, USA).

Table 2. Primer sequences used for qRT-PCR analysis (AT, annealing temperature (°C)).

Genes	Primer sequence (5'–3')		References	AT
	Forward	Reverse		
β-actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	NM-001101	63
Claudin-3	CTGCTCTGCTGCTCGTGTC	CGTAGTCCTTGCGGTCGTAG	NM-001306	63
Claudin-4	GTCTGCCTGCATCTCCTCTGT	CCTCTAAACCCGTCCATCCA	NM-001305	62.5
E-cadherin	TGGACCGAGAGAGTTTCCCT	CCCTTGTACGTGGTGGGATT	BC-144283.1	60
Occludin	TTGGATAAAGAATTGGATGACT	ACTGCTTGCAATGATTCTTCT	NM-002538	57
ZO-1	GAATGATGGTTGGTATGGTGCG	TCAGAAGTGTGTCTACTGTCCG	NT-010194.17	55.8
IL-6	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTT	S56892.1	63
IL-8	CTCTTGGCAGCCTTCCTGATT	TATGCACTGACATCTAAGTTCTTTAGCA	NM-000584.3	60
IL-1β	GCTGAGGAAGATGCTGGTTC	GTGATCGTACAGGTGCATCG	NM-000756	57

Membranes were blocked in blocking buffer (0.05% (v/v) Tween-20 in PBS (PBST) and 5% (w/v) milk proteins) for 1 h at room temperature. The membranes were incubated overnight (at 4 °C) with primary antibodies of CLDN-3 and 4, ZO-1 (341700, 329400 and 402200, Invitrogen, Carlsbad, CA, USA), OCLD (AB31721, Abcam, Cambridge, UK) and E-cadherin (610182, eBioscience, San Diego, CA, USA) diluted in blocking buffer according to manufacturer's instructions. After washing in PBST for 3 times, the membranes were incubated with appropriate secondary antibodies (Dako, Glostrup, Denmark) for 2 h at room temperature. Blots were washed in PBST and incubated with ECL reagents mix (Amersham Biosciences, Roosendaal, The Netherlands) and the bands were visualized using ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Image Lab software version 5.2 (Bio-Rad Laboratories, Hercules, CA, USA). Monoclonal rabbit anti-human β-actin antibody (Cell Signaling, Danvers, MA, USA) was used to evaluate homogeneity of loading and normalize the optical density of the bands. The protein levels were expressed as mean fold change in relation to the control group.

Immunofluorescence staining

Immunofluorescence staining was conducted to determine cellular localization of ZO-1, OCLD, CLDN-1, 3 and 4 and E-cadherin. BeWo cells were grown on 24-wells plates and exposed to different mycotoxins at the apical compartment for 24 h. The cells were fixed with 10% formalin and washed with PBS prior to permeabilization with 0.1% (v/v) Triton-X-100 solution for 5-10 minutes. Samples were blocked for 30 minutes at room temperature using 5% serum diluted in PBS containing 1% BSA. Thereafter, cells were incubated for 2 h at room temperature with primary antibodies against CLDN3, CLDN4, OCLD, ZO-1 (341700, 329400, 331500 and 402200, Invitrogen, Carlsbad, CA, USA) or E-cadherin (610182, eBiosciences, San Diego, CA, USA) diluted in 1% PBS- BSA solution,

according to manufacturer's instructions. After washing, cells were incubated with Alexa-Fluor conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Anti-fading mounting medium containing DAPI (Prolong gold with DAPI, Life technologies, Thermo Fisher Scientific, Wilmington, DE, USA) was used for nuclear counterstaining and sealing the wells with a coverslip. Localization of the junctional proteins was visualized using the Microscope Leica TCS SP8 X**.**

Quantitative determination of interleukin-6 (IL-6), IL-8 and IL-1β

The concentration of IL-6 in the cell culture supernatants was determined after 24 h of mycotoxin exposure in BeWo cells, using the commercially available human IL-6, IL-8 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, San Diego, CA, USA) following the guidelines provided by the manufacturer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 7.04, La Jolla, CA, USA). Results are expressed as mean ± SEM and differences between groups are statistically determined by using one-way analysis of variance (ANOVA). When significant differences were observed, Bonferroni post hoc analysis was used to identify significant differences between the means. Differences were considered significant at $P < 0.05$. Different lower-case letters on the bars indicates significant differences between groups and letters shared in common between or among the groups would indicate no significant difference.

Results

Concentrations of 8 µM DON, 16 µM ZEN and 8 nM T-2 toxin are cytotoxic for Bewo cells.

The direct cytotoxic effects of DON, ZEN and T-2 toxin on BeWo cells were assessed by measuring lactate dehydrogenase (LDH) release in cell supernatants after 24 h exposure. The LDH levels were increased after exposure to increasing concentrations of DON, ZEN or T-2 toxin. Concentrations of 8 µM, 16 µM and 8 nM of DON, ZEN and T-2 toxin, respectively, led to significantly higher LDH release, compared to the control (Figure 1). In all subsequent experiments, concentrations equal to or below these toxic levels of mycotoxins were used.

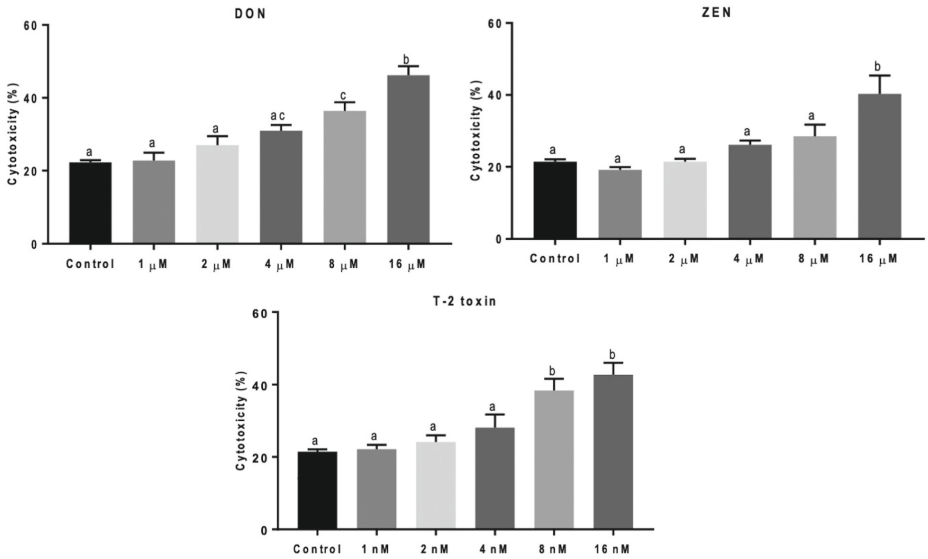

Figure 1. Lactate dehydrogenase (LDH) release in BeWo cells incubated for 24 h with increasing concentrations of deoxynivalenol (DON), zearalenone (ZEN) and T-2 toxin. Data are representative of three independent experiments, each performed in triplicate, and expressed as the mean ± SEM. Different lowercase letters denote significant differences among the treatments ($p < 0.05$).

Mycotoxins exposure disrupts the integrity and increases permeability of the BeWo cell layer

The integrity of the cellular monolayer after mycotoxins exposure was evaluated by adding increasing concentration of mycotoxins to the apical compartments of Transwell inserts. Transepithelial electrical resistance (TEER) was significantly decreased after 24 h exposure to DON and T-2 toxin, while ZEN had no effect on TEER values (Figure 2A). Correspondingly, a significant increase in paracellular transport of fluorescein isothiocyanate-dextran (FITC-D) across the cell monolayer from the apical to the basolateral chamber after DON and T-2 toxin exposure was observed; but, after ZEN exposure, the paracellular transport of FITC-D was unaffected (Figure 2B).

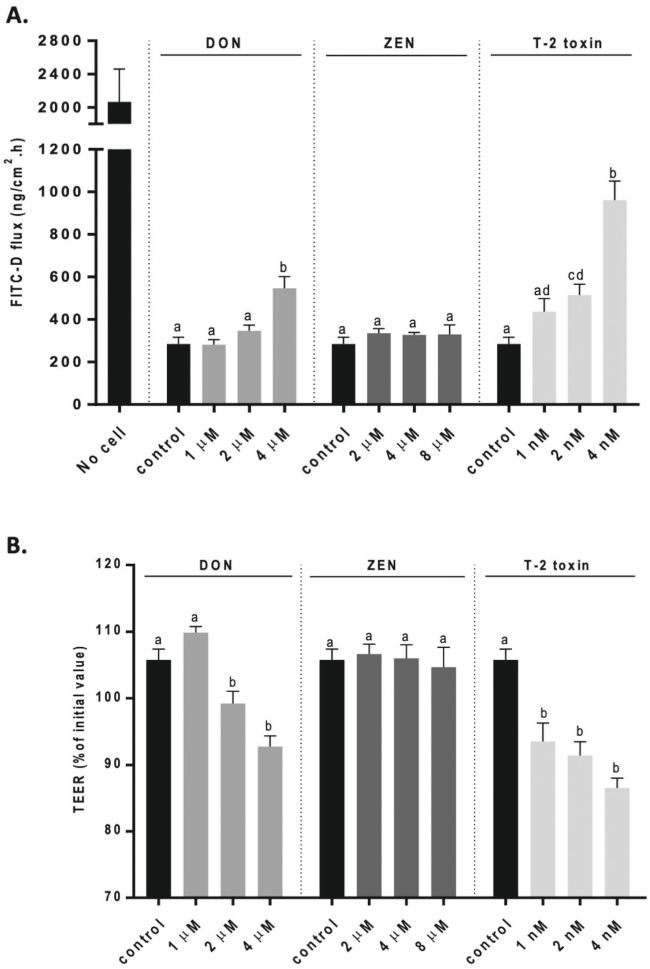

Figure 2. Effects of deoxynivalenol (DON), T-2 toxin, and zearalenone (ZEN) exposure on the integrity of the BeWo cell monolayer. The changes in (A) transepithelial electrical resistance (TEER) and (B) fluorescein isothiocyanate-dextran (FITC-D) flux from apical to basolateral compartment after 24 h exposure to mycotoxins at the apical side are shown. Data are expressed as the mean ± SEM of three independent experiments, each performed in triplicate. Different lowercase letters denote significant differences among groups. ($p < 0.05$).

Mycotoxins exposure alters gene expression of junctional proteins

In order to assess the effect of 24 h exposure to different mycotoxins on barrier function of BeWo cells in more detail, mRNA expression levels of different junctional proteins, including occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, and E-cadherin, were assessed by Real-Time Polymerase Chain Reaction (qPCR) analysis.Exposure to the low concentrations of DON (2 and 4 µM) resulted in increased mRNA levels of ZO-1 and OCLD, but no significant effect on CLDN-3 and CLDN-4 mRNA

levels were observed (Figure 3). All DON concentrations significantly decreased the mRNA expression of E-cadherin. *The lower concentrations of ZEN showed no significant effect on mRNA level of all tight junctions (TJ) and E-cadherin (Figure 4), but the higher concentrations caused a reduction in mRNA levels of ZO-1, CLDN-4 and E-cadherin. Exposure to T-2 toxin resulted in a similar mRNA-expression pattern as observed for cells exposed to DON (Figure 5). concentrations of 1 and 2 nM of T-2 toxin significantly increased mRNA level of ZO-1 and OCLD, but had no significant effect on CLDNs. The lower concentrations of T-2 toxin had no effect on E-cadherin mRNA expression, but the higher concentrations caused significant decrease.*

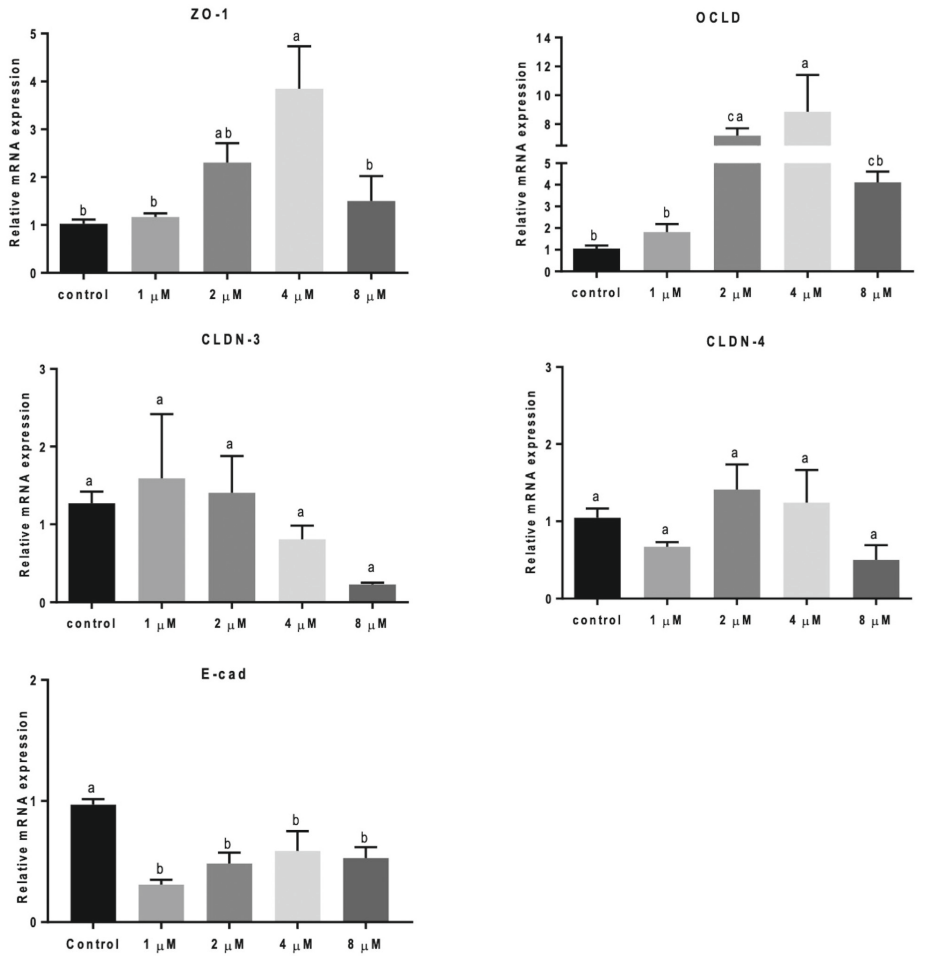

Figure 3. Effects of deoxynivalenol (DON) exposure on mRNA levels of junctional proteins in BeWo cells; The mRNA expression of occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, E-cadherin (E-cad) in BeWo cells after 24 h exposure to different concentrations of DON. Data are expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences between groups ($p<0.05$).

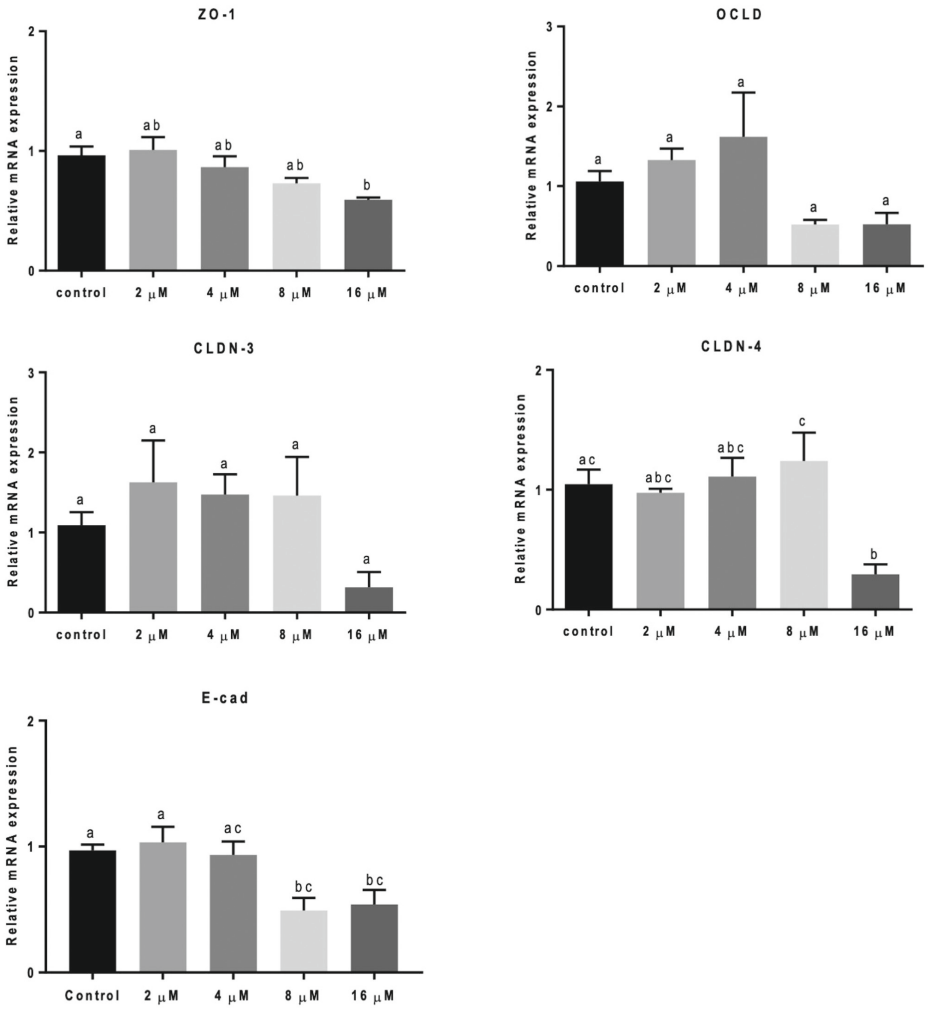

Figure 4. Effects of zearalenone (ZEN) exposure on mRNA levels of junctional proteins in BeWo cells; The mRNA expression of occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, E-cadherin (E-cad) in BeWo cells after 24 h exposure to different concentrations of ZEN. Data are expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences between groups ($p<0.05$).

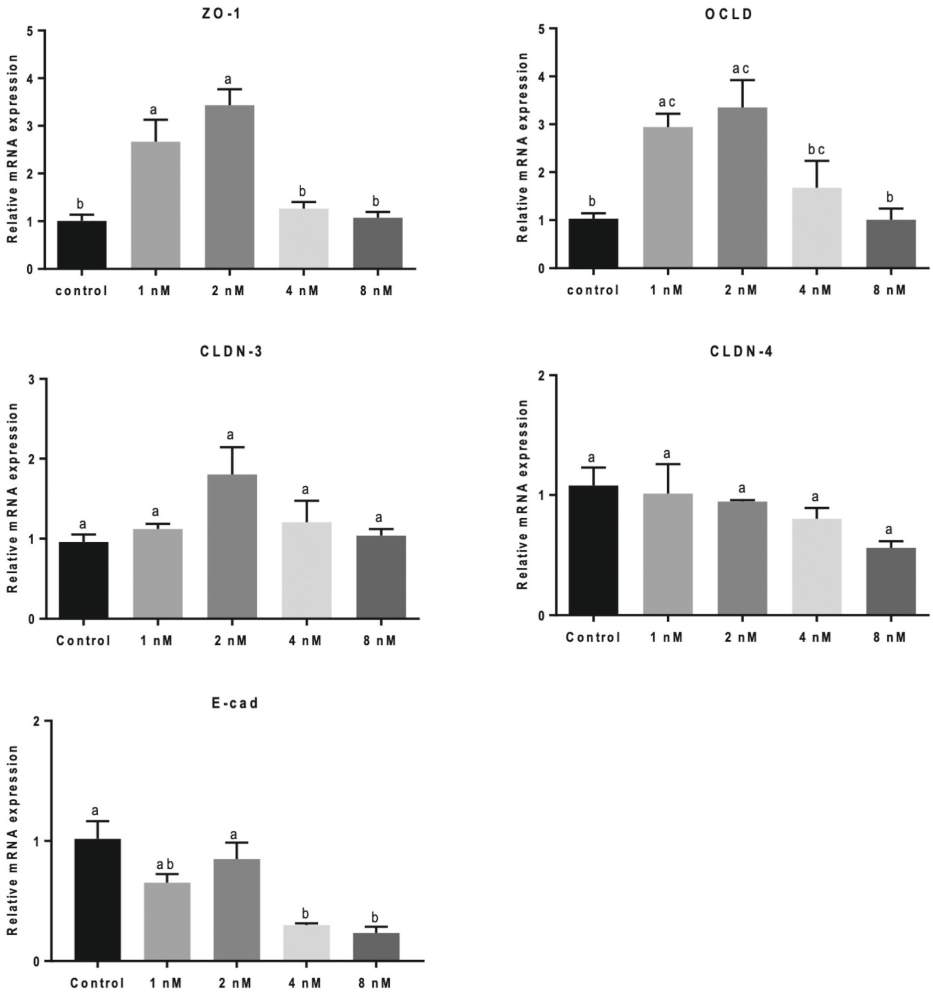

Figure 5. Effects of T-2 toxin exposure on mRNA levels of junctional proteins in BeWo cells; The mRNA expression of occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, E-cadherin (E-cad) in BeWo cells after 24 h exposure to different concentrations of T-2 toxin. Data are expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences between groups ($p<0.05$).

Mycotoxins exposure modulates the protein expression of junctional proteins

Protein levels of TJs and E-cadherin in mycotoxin-exposed BeWo cells were assessed with western blot analysis. BeWo cells were collected after 24 h exposure to increasing concentrations of DON, ZEN and T-2 toxin. The lower concentrations of DON increased the protein level of ZO-1, and had no effect on CLDN-3, CLDN-4 and E-cadherin, but higher concentrations caused significant reduction in expression of ZO-1, CLDNs and

E-cadherin proteins. The expression of OCLD was not significantly affected by DON (Figure 6). As shown in figure 7, the effect of ZEN on protein levels of TJs was not clear and only the higher concentration of ZEN decreased the expression of OCLD and E-cadherin, but these effects were not statistically significant. Expression of ZO-1 and E-cadherin were significantly reduced after exposure to T-2 toxin. CLDN-3 and 4 protein levels were only significantly reduced by the higher concentration of 8 nM, and the expression of OCLD protein was not significantly affected (Figure 8).

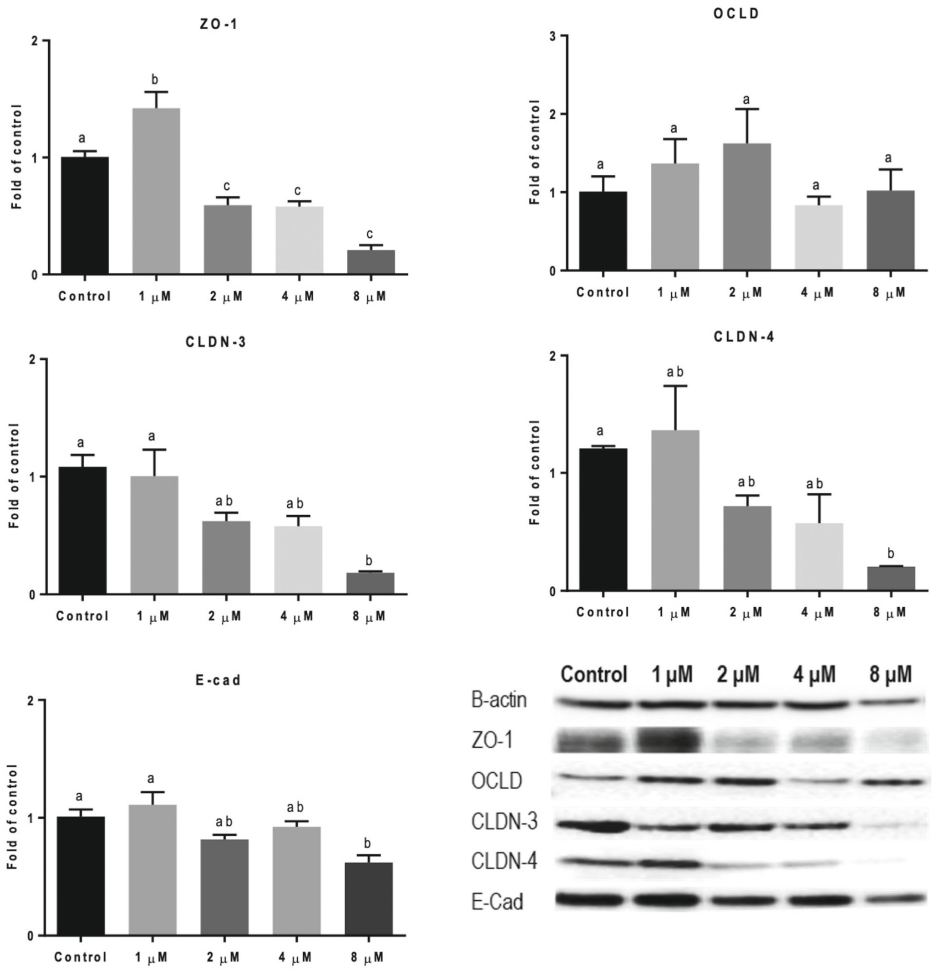

Figure 6. Effects of deoxynivalenol (DON) exposure on junctional proteins in BeWo cells; The protein expression of occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, E-cadherin (E-cad) in BeWo cells after 24 h exposure to different concentrations of DON. Data are expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences between groups ($p<0.05$).

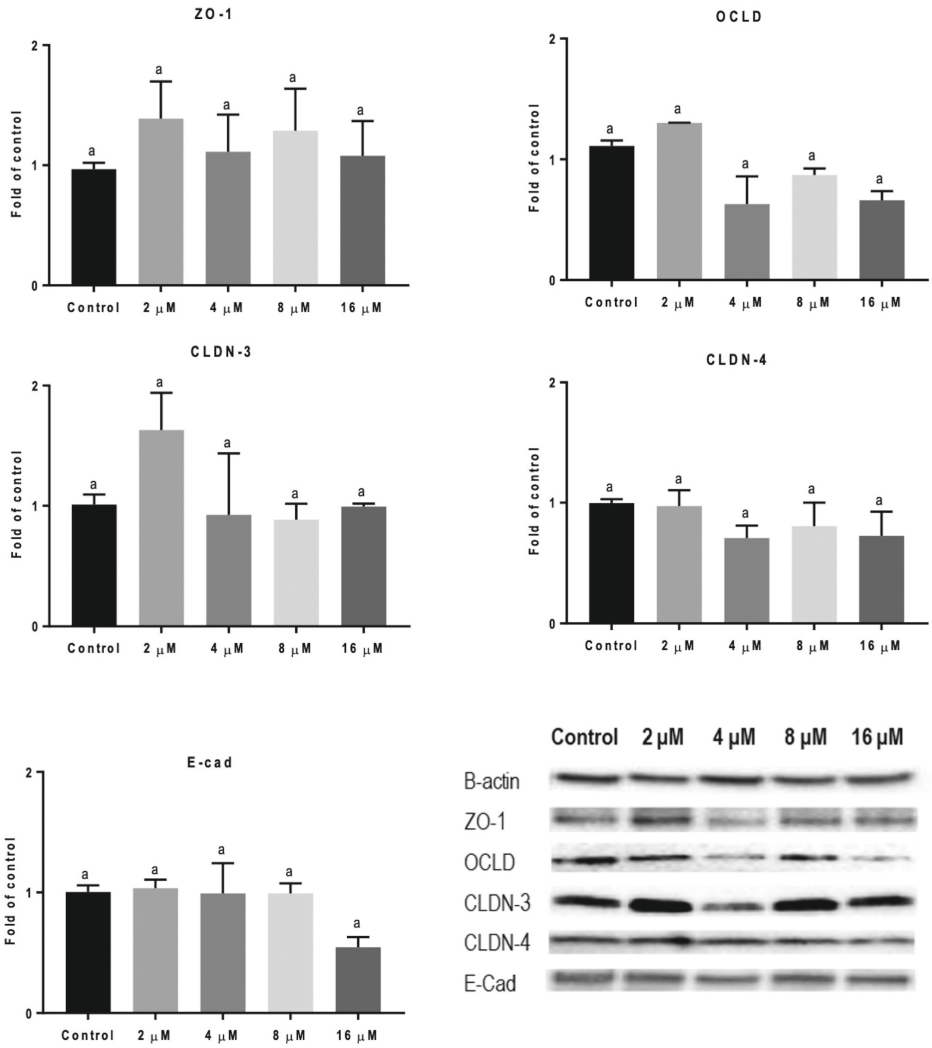

Figure 7. Effects of zearalenone (ZEN) exposure on junctional proteins in BeWo cells; The protein expression of occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, E-cadherin (E-cad) in BeWo cells after 24 h exposure to different concentrations of ZEN. Data are expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences between groups ($p<0.05$).

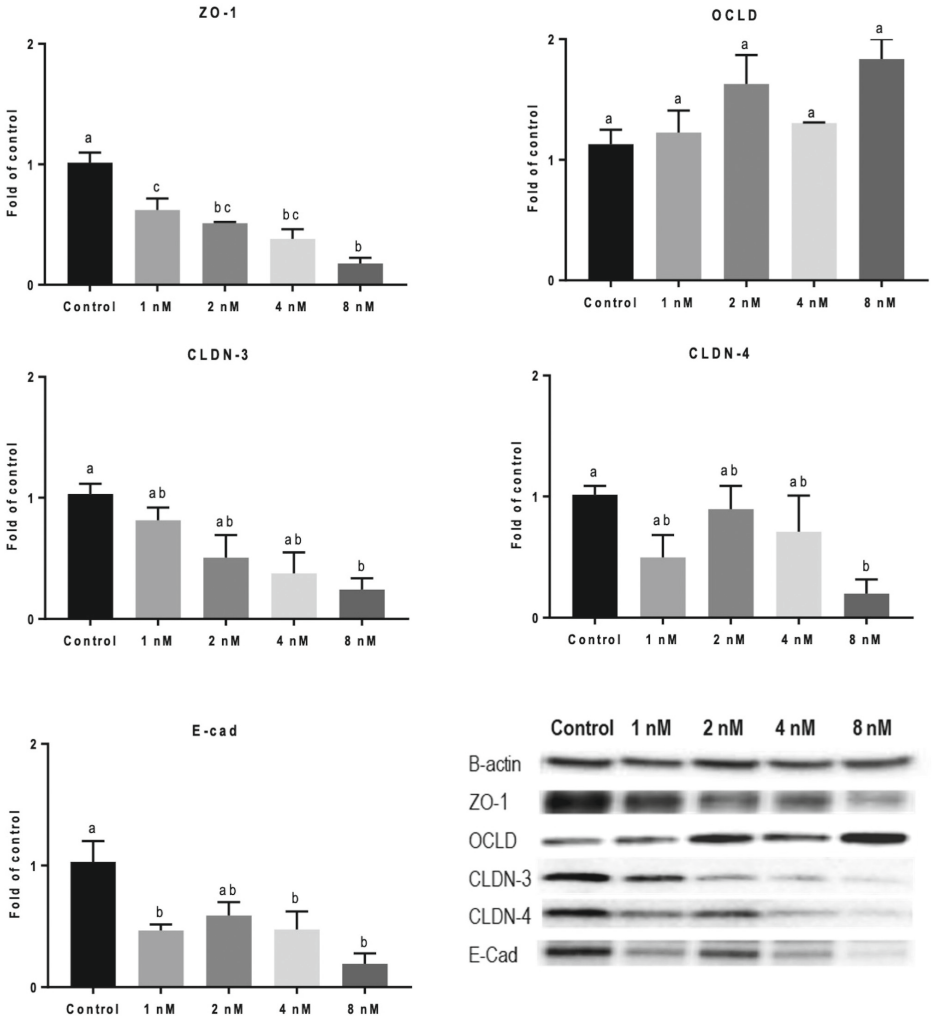

Figure 8. Effects of T-2 toxin exposure on junctional proteins in BeWo cells; The protein expression of occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, E-cadherin (E-cad) in BeWo cells after 24 h exposure to different concentrations of T-2 toxin. Data are expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences between groups ($p<0.05$).

Mycotoxins exposure alters the localization of junctional proteins

To investigate the cellular localization of junction proteins, immunofluorescence staining was performed using BeWo cells grown on 24-well plates incubated with or without increasing concentrations of DON, ZEN or T-2 toxin for 24 h. In the intact BeWo cells OCLD, CLDN-4, ZO-1 and E-cadherin were localized at the cell membrane and formed continuous belt-like structures (Figures 9 and 10). DON and T-2 toxin exposure increased

delocalization and intracellular accumulation of OCLD (Figure 9A), and decreased expression and induced a disturbed and irregular cellular distribution of CLDN-4 protein on the cell surface, especially by the higher concentrations (figure 9B). The higher concentration of ZEN (16 µM) also caused similar modifications on CLDN-4 and OCLD, but the effect is less intense. The higher DON and T-2 toxin concentrations caused an obvious irregular assembly of E-cad and ZO-1 proteins, depicted as discontinuous belt-like structures around the cells. The ZEN exposure showed no visible effect on these two proteins (Figures 10A and B).

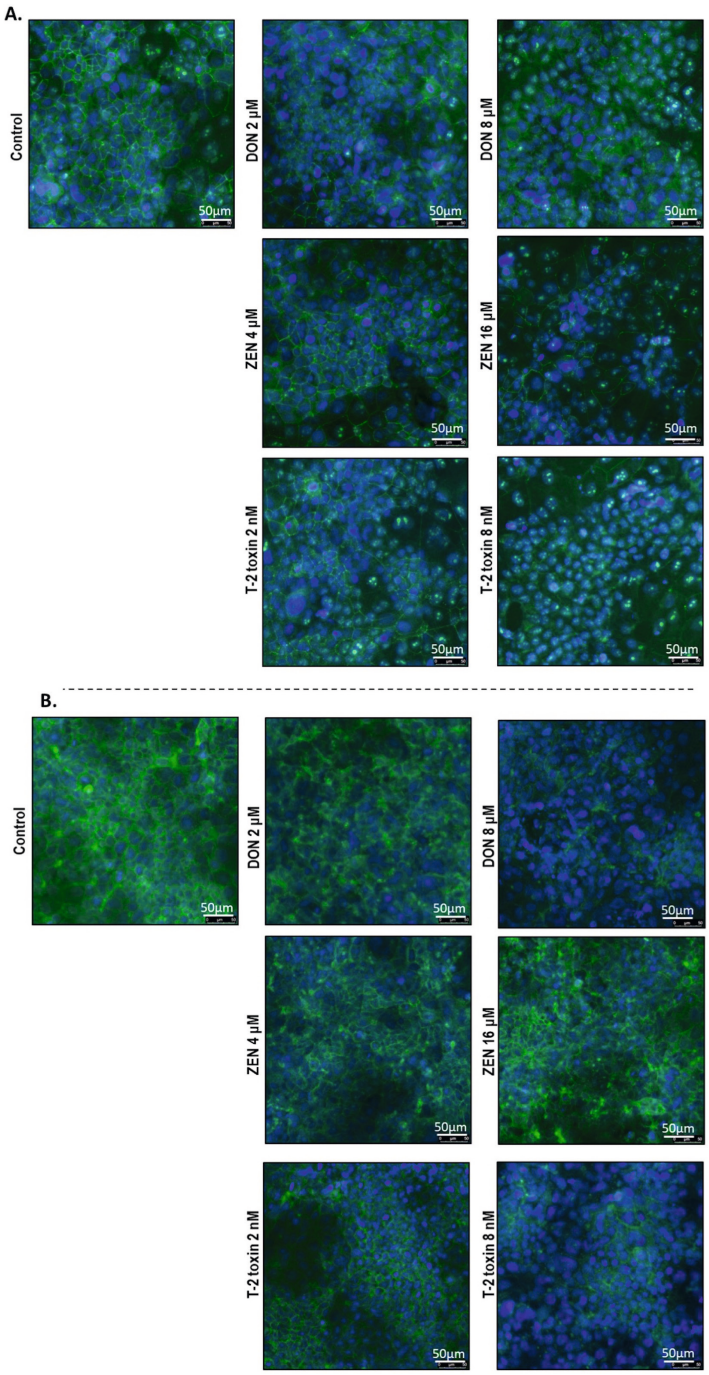

Figure 9. Effects of deoxynivalenol (DON), T-2 toxin, and zearalenone (ZEN) exposure on (A) occludin (OCLD) and (B) claudin (CLDN)-4 localization in BeWo cells, visualized by immunofluorescence staining (400× magnification). Scale bars represent 50 µm.

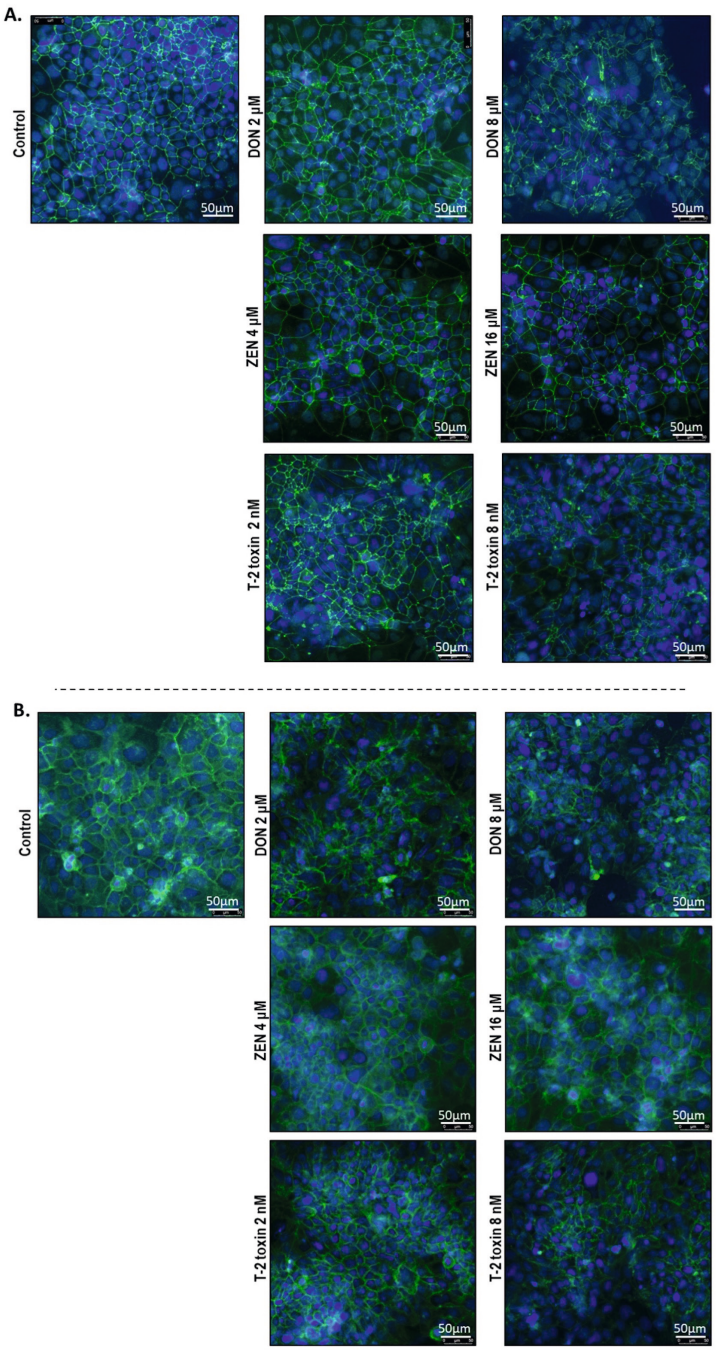

Figure 10. Effects of deoxynivalenol (DON), T-2 toxin, and zearalenone (ZEN) exposure on (A) occludens protein-1 (ZO-1) and (B) E-cadherin localization in BeWo cells, visualized by immunofluorescence staining (400× magnification). Scale bars represent 50 µm.

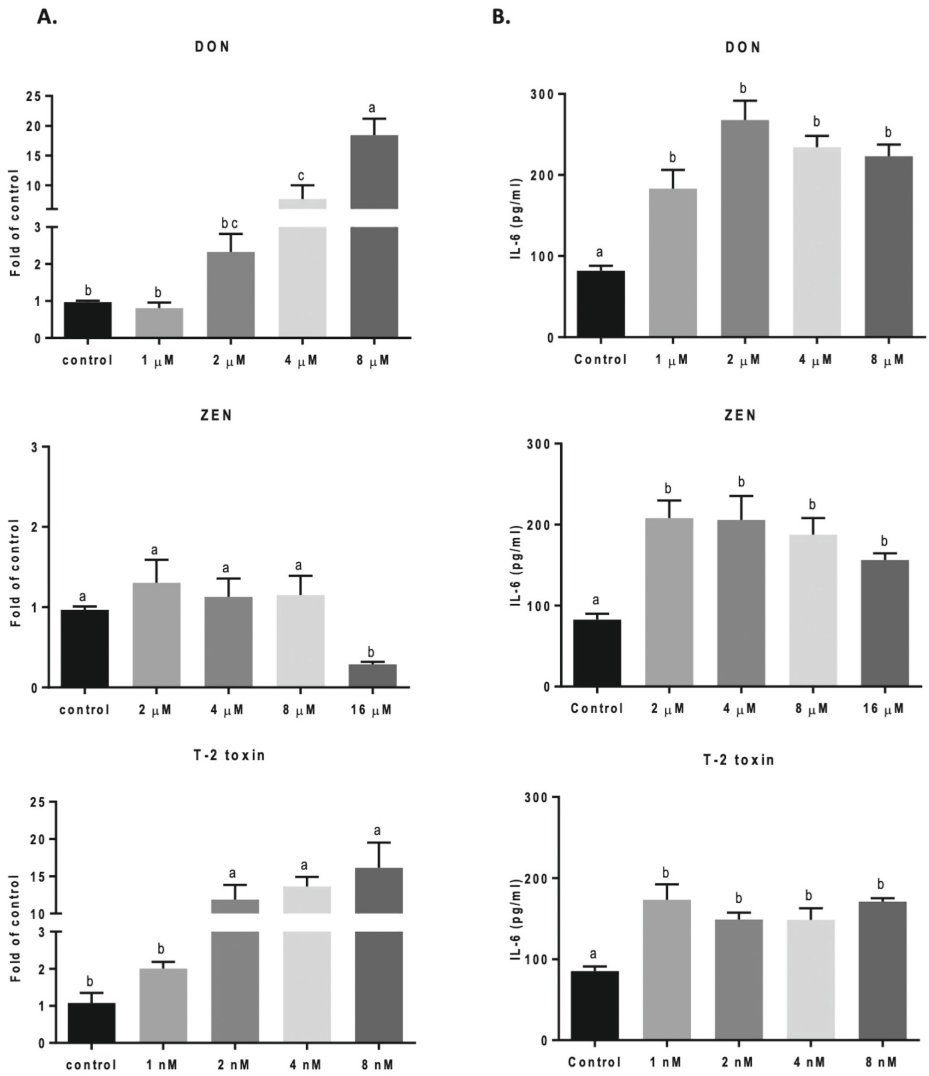

Figure 11. Effects of mycotoxin exposure on IL-6 mRNA expression and IL-6 secretion in BeWo cells. BeWo cells were incubated for 24 h with increasing concentrations of deoxynivalenol (DON), zearalenone (ZEN) and T-2 toxin, and the interleukin (IL)-6 mRNA expression (A) as well as secretion (B) were measured. Data are expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences between groups ($p<0.05$).

Mycotoxins exposure increases interleukin-6 (IL-6) mRNA expression and protein release.

Exposure to DON and T-2 toxin caused a significant increase in mRNA level of IL-6 (Figure 11A), and led to significantly higher levels of this cytokine in the cell supernatant compared to the untreated cells (Figure 11B). Although ZEN had no significant effect on mRNA expression of IL-6, the cytokine release was increased, even with the lower concentrations of ZEN. However, none of the tested mycotoxins stimulated IL-8 and IL-1β cytokine release or mRNA expression (data not shown).

Discussion and conclusion

The placental epithelium covers the maternal surface of the placenta, and forms a polarized layer of cells, where the microvillous membrane of the syncytiotrophoblasts is in direct contact with the maternal blood, while the basal membrane faces the fetal circulation [38]. This cell layer forms a semipermeable barrier by restricting the paracellular space with junctional proteins [39-41]. The placenta is essential for protecting and nourishing the fetus during pregnancy and has profound consequences for life-long health. Disruption of junctional proteins in placenta as a result of maternal exposure to toxic dietary contaminants can lead to impaired maternal-fetal barrier and endanger the growth and health of the fetus.

Mycotoxins are natural food and feed contaminants, which can significantly impact human and animal health. Limited studies report on the effects of mycotoxins on placental epithelium, therefore, the current study focused on the direct effect of exposure to 3 major *Fusarium* mycotoxins on barrier and immune function of human placental cells. There is scarce information about the exact serum levels of mycotoxins in different populations [42, 43]; however, these dietary contaminants can be present in a wide range of food products, and the amount and the type of daily food intake varies for every individual. Therefore, in order to set proper limitations on the mycotoxin exposure levels during pregnancy, it is important to determine the minimum concentrations that can impose toxic effects on human placental cells.

Exposure to increasing concentrations of mycotoxins for 24 h caused concentration-dependent cell toxicity in BeWo cells. The T-2 toxin exposure caused cellular leakage at much lower concentrations in comparison to the other toxins, whereas ZEN could be ranked as the least toxic mycotoxin among the three. The concentration of T-2 toxin in naturally contaminated grains and cereals and the average daily exposure to this toxin are usually lower than the other Fusarium mycotoxins [44]. However, due to its high toxicity, it has a very low TDI level in humans compared to DON and ZEN (1, 0.25 and 0.02 µg/kg body weight/day for DON, ZEN and T-2 toxin, respectively) [45-48], and based on the data from this study, exposure to very low concentrations of T-2 toxin can cause significant toxicity on BeWo cells. In this study, exposure to individual Fusarium

mycotoxins is investigated. However, simultaneous exposure to different mycotoxins, which occurs naturally as a result of either contemporary production of different toxins from some fungal species or mixed fungi infections in commodities, is raising concerns about the possible additive or synergistic toxic effects [49]. The co-ncurrence of low doses of different mycotoxins in food –especially the trichothecenes– may be even more toxic than predicted for the individual mycotoxins [50]. This underlines the importance of studying the effects of these toxins on vulnerable tissues such as the placenta.

The significant decrease in electrical resistance and increase in intracellular permeability of FITC-D after exposure to DON and T-2 toxin indicates the detrimental effect of these two mycotoxins on barrier function and integrity of placental epithelial cell line. Previous studies reported no effect of T-2 toxin [34] and DON [51] exposure on BeWo cells after 6 and 12 hours, respectively; however, according to the results of this study longer exposure (24 h) to the lower concentrations of these mycotoxins can significantly disrupt barrier integrity. Intercellular junctions play a crucial role in formation and maintenance of epithelial barriers [52]. Exposure to DON or T-2 toxin changes the mRNA and protein expression patterns of junctional proteins in BeWo cells. An up-regulation of the mRNA expression of OCLD and ZO-1 by lower concentrations of DON and T-2 toxin was observed, while the mRNA expression of E-cadherin was significantly decreased by all concentrations of DON (Table 1). The increase in mRNA levels of ZO-1 is associated with the reduction in protein expression of ZO-1 and indicates a compensatory response to the stress induced by non-cytotoxic levels of DON and T-2 toxin [37, 53]. In addition, due to the involvement of ZO-1 in cadherin-based cell-cell adhesion, it can be speculated that the increase in the mRNA expression of ZO-1 might be partly associated with the reduction of E-cadherin in protein level [54, 55]. However, for CLDN-3 and 4, the observed reduction in protein expression was not accompanied by prominent changes in mRNA expression. The underlying mechanism responsible for these changes in expression patterns needs further investigation in order to unravel the differences between the mycotoxins. DON and T-2 toxin both belong to the group of trichothecenes and impose their toxic effect by generating the production of free radicals, and inducing the lipid peroxidation which lead to the imbalance of the antioxidant status of the cells [56]. Moreover, mitochondria-related caspase-dependent apoptotic pathway can be involved in DON induced-cytotoxicity [57]. DON and T-2 toxin can bind to the ribosomes and activate mitogen-activated protein kinases (MAPKs) through the initiation of ribotoxic stress response and induce protein oxidation and apoptosis [56], which could be a possible mechanism involved in the observed effects on TJ expression by DON and T-2 toxin. In this regard, it has been shown that induction of MAPK activation by DON treatment can modulate the expression of claudin-4 in an intestinal epithelial cell line [58].

Exposure to ZEN had less prominent effects on the mRNA and protein expression of TJs, which may partially explain the unchanged TEER and FITC-D permeability in BeWo

cell layer after 24 h exposure. High concentrations of ZEN decreased the mRNA levels of TJs but had no significant effect on their protein expressions (Table 1). Structural and functional differences between ZEN and the other two mycotoxins might be responsible for the difference in toxic effects of these mycotoxins. ZEN is a non-steroidal estrogenic mycotoxin [59]. It can interact with estrogenic receptors and disturb the cell cycle progression, thereby inhibiting cell proliferation [59]. Longer exposure to ZEN can trigger the differentiation of BeWo cells into syncytiotrophoblast-like cells, inducing morphological changes as well as human chorionic gonadotropin (hCG) secretion [15]. Possibly, the induction of differentiation in BeWo cells may partially be responsible for the observed effects of ZEN on TJ expression [60].

The integrity of the BeWo cell layer is not only maintained by sufficient expression levels of junctional proteins; their cellular localization and distribution are also of critical importance. Mycotoxins DON and T-2 toxin caused reduction and fragmentation of the junctional network of ZO-1, E-cad and CLDN-4 (Table 1), which corresponded to the observed reduction in protein expression levels of these junctional proteins. Furthermore, an irregular accumulation of OCLD proteins in cells exposed to DON and T-2 toxin was observed. The effect of ZEN on TJ protein assembly was less pronounced. However, after exposure to the highest concentration of ZEN, aberrant structures and membrane dislocations of CLDN-4 and OCLD were observed. Abnormally localized OCLD protein may explain the increased levels of OCLD mRNA expression as a repair mechanism in response to the lack of OCLD protein in the cell membrane. Our observations indicate that these mycotoxins can disrupt the placental barrier, which in the *in vivo* situation may facilitate the transfer of harmful chemicals and toxins through the placenta. Several *in vivo* and *in vitro* studies provide evidence on transfer of mycotoxins from maternal blood into the fetal side, where the unchanged and metabolized forms of these toxins can be detected in fetal samples [8, 33, 61]. Our results show that this may be due, at least in part, to the impaired junctional protein networks of the trophoblast cell layer.

The observed adverse effects of the tested mycotoxins on placental epithelial integrity can be due to either their direct effect on the cells, or via induction of inflammatory responses. Human trophoblasts are capable of expressing a number of cytokines, including IL-6, which in non-pathologic conditions play an important role in maintenance of pregnancy [62, 63]. However, pathologic conditions can alter the cytokine release pattern and enhance IL-6 release [64]. It has been shown that IL-8 production and IL-6 transcription and secretion were concentration-dependently enhanced in BeWo cells, after IL-1 and tumor necrosis factor (TNF)-α stimulation, however, transforming growth factor (TGF)-β stimulation caused elevated IL-6 levels without affecting IL-8 production [65]. In the current study, we showed that DON, T-2 toxin and ZEN increase the production of IL-6 in BeWo cells, even at low concentrations, but they do not induce IL-8 release. These observations suggest that tested mycotoxins may stimulate IL-6

production via the pathways similar to TGF-β induction. It has been suggested that interleukins can act as modulators of junctional complexes, including those of the placenta [29], and alter the permeability characteristics of epithelial barriers, affecting OCLD, CLDNs and ZO-1 proteins [66]. IL-6 can increase paracellular permeability and change the distribution and morphology of TJ protein assembly in endothelial cell layer [67]. IL-6 increases TJ permeability of intestinal epithelium by altering the expression of claudin-2 protein via an IL-6Rα-coupled signal transducer and gp130 signaling pathway [68]. It has been shown that untreated BeWo cells can express both IL-6R and gp130 mRNA [69]. Therefore, increased IL-6 production in BeWo cells may account for the detrimental effects of the tested mycotoxins and can be partially responsible for the observed irregular localization of the junctional proteins. Apart from its effect on TJs, IL-6 can impose adverse effects on placental transportation through other pathways as well. High levels of IL-6 in maternal blood can stimulate trophoblast fatty acid accumulation, and consequently contribute to an excessive nutrient transfer across the maternal-fetal barrier [70]. In addition, IL-6 was shown to be transferred to the fetus both in mid and late gestation after intravenous administration in pregnant rats [71]. Prenatal exposure to IL-6 in early and late pregnancy, can lead to long-term adverse effects including insulin resistance, elevated stress response, hypertension and dysregulation of hypothalamic-pituitary-adrenal axis activity during adulthood [72, 73].

In this study, IL-6 mRNA expression levels were concentration-dependently increased by exposure to DON and T-2 toxin, but not by ZEN. As mentioned earlier, ZEN may trigger BeWo cell trophoblast differentiation [15] which is accompanied by a marked decrease in mRNA expression of some interleukins, including Il-6 [74].

In conclusion, our study is the first to report that major Fusarium mycotoxins can increase paracellular permeability in BeWo cells by inducing significant changes in expression and delocalization of junctional proteins. Furthermore, the production of IL-6 was increased in response to mycotoxin exposure, which can induce detrimental effects on fetal development, and might be partially responsible for the mechanisms involved in altered structure of junctional network. Although ZEN did not induce any obvious effects on barrier function, it significantly increased the IL-6 production, suggesting that mechanisms other than those involving IL-6 might be responsible for observed mycotoxin-induced damages (Table 1). Therefore, further investigations are required to understand the exact mechanism underlying the toxicity of these mycotoxins on the placental barrier. In this study, the effects of direct exposure of major Fusarium mycotoxins to the placenta epithelial cell line were investigated, and as we observed significant changes in BeWo monolayer permeability, inflammatory responses and structural changes in the cells, it can be inferred that mycotoxins may bring about detrimental consequences on fetal health and cause an imbalanced transportation of nutrients; therefore, the results from our study emphasize the necessity to further investigate potential detrimental consequences on fetal health.

Table 1. Summary effects of deoxynivalenol (DON), zearalenone (ZEN), and T-2 toxin on occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, and E-cadherin (E-cad).

	TJ or AJ	mRNA	Protein	IF staining
Mycotoxin DON	ZO-1	↑	↓	Decreased and irregular assembly
	OCLD	↑	–	Increased delocalization and intracellular accumulation
	CLDNs	–	↓	Decreased and modified assembly and belt-like structure
	E-cad	↓	↓	Decreased and irregular assembly
Mycotoxin ZEN	ZO-1	↓	–	No obvious effect
	OCLD	–	–	Increased delocalization and intracellular accumulation
	CLDNs	↓	–	Modified assembly and belt-like structure
	E-cad	↓	–	No obvious effect
Mycotoxin T-2 toxin	ZO-1	↑	↓	Decreased and irregular assembly
	OCLD	↑		Increased delocalization and intracellular accumulation
	CLDNs	–	↓	Decreased and modified assembly and belt-like structure
	E-cad	↓	↓	Decreased and irregular assembly

Author Contributions

Conceptualization: S.B., A.H.; methodology, S.B., S.V.; validation, N.S.T., S.V.; formal analysis, N.S.T.; investigation, N.S.T.; resources, J.G., B.v.t.L., G.F., A.D.K.; writing-original draft preparation, N.S.T.; writing-review and editing, S.B., A.H., S.V., B.v.t.L, G.F., A.D.K., J.G.; visualization, N.S.T., S.B.; supervision, S.B., A.H.; project administration, S.B., A.H.; funding acquisition, J.G., B.v.t.L.

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Chapter 5

Deoxynivalenol exposure during pregnancy has adverse effects on placental structure and immunity in a mice model

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Abstract

Deoxynivalenol (DON), a highly prevalent food contaminant, is known to induce effects on reproduction as well as immunity in humans upon exposure. The present study focused on the consequences of exposure to DON during pregnancy for placental barrier and immune function, as well as fetal survival. Female mice received diets contaminated with DON (6.25 and 12.5 mg/kg of diet), starting immediately after mating until the end of the experiment. On day 17 of pregnancy the animals were euthanized, and maternal and fetal samples were collected for further analysis. Feeding on DON-contaminated diets decreased fetal survival, and DON was detected at significant levels in the fetus. Placentae from DON-exposed mice revealed a reduction in expression of junctional proteins, ZO-1, E-cadherin and claudins, upregulation of AHR mRNA expressions, and increase in IFN-γ, IL-6 and IL-4 production. In conclusion, results of this study demonstrate harmful effects of DON on the course of pregnancy and fetal survival, which might be due to immunological changes in maternal immune organs and placenta. Altogether, these data underline the importance of the quality of maternal diet during pregnancy as they clearly demonstrate the potential harmful effects of a commonly present food-contaminant.

Introduction

Pregnancy is a critical period of physiological changes for both the mother and the fetus. Lifestyle, including maternal nutrition, is crucial in long-term health of the infant. Beneficial effects of nutritional supplementation during pregnancy with, for example, prebiotics and polyunsaturated fatty acids on the development of the fetus have been demonstrated previously [1, 2]. On the other hand, exposure to various environmental factors and contaminants can interfere with the process of developmental programming, leading to long-term or permanent structural or functional changes in the offspring. Exposure to such stimuli can potentially impose adverse effects on fetal survival and health [3]. The exact mechanisms and causes of many adverse pregnancy outcomes are not fully understood yet, but increasing evidence links pregnancy complications and incidence of some non-communicable chronic diseases in the offspring to the quality of maternal nutrition [4-6]. Moreover, the developing fetus is vulnerable to a wide range of contaminants in maternal food and the environment, which can reach the fetus during pregnancy and lead to adverse developmental outcomes [4, 7], including childhood cancers [8], allergic and autoimmune diseases [9, 10].

An important group of such nutritional contaminants are mycotoxins, which are secondary metabolites of fungi. Some of the highly prevalent mycotoxins such as aflatoxins, ochratoxins, fumonisins, trichothecenes and zearalenone can induce acute or chronic toxicity upon consumption in humans and animals, even at low concentrations [11]. These mycotoxins are produced by fungal species in the Aspergillus and Fusarium genera, which most frequently contaminate agricultural food products, especially cereal crops [11, 12]. As a result of the high prevalence of these fungi in the food chain, mycotoxin exposure is almost ubiquitous and epidemiological studies from different geographical regions indicate that pregnant women and newborns can be easily exposed to mycotoxins [7, 13, 14]. This potentially harmful exposure during the critical period of pregnancy and early-life is associated with various adverse health outcomes, such as skeletal malformation, neural tube defects, growth retardation and neonatal jaundice [15, 16].

Deoxynivalenol (DON), a trichothecene mycotoxin produced by various different Fusarium species, is one of the most common contaminants of cereal crops, especially oats, barley, wheat and maize [17]. Human exposure to DON has been indicated to frequently exceed the established safety levels [18-20]. Masked and modified forms of DON, which are not detected by established standard methods, may lead to underreporting of mycotoxin levels in food products [21]. Thus, investigating the toxicity of DON exposure on human health is of utmost importance. The mechanisms by which DON causes toxicity are induction of oxidative stress, DNA fragmentation and inhibition of protein synthesis [22, 23]. Exposure to DON elicits pro-inflammatory responses, cytotoxicity and apoptosis in different organs [24-26], which can result in

growth impairment and impose adverse effects on gastrointestinal tract, immune regulation and reproduction [27]. In several animal species, DON exposure induced reproductive toxicity and teratogenic effects [28]; the most prominent consequences for the fetus were abnormal skeletal development and lower fetal survival rate [29-31].

Considering that the placenta is the only crossing point between maternal and fetal circulation, fetal exposure to DON can only occur via placental transport. Indeed, DON can be transcellularly transported across BeWo cell monolayers, a trophoblastic cell line used to model the human placental barrier [32]. Moreover, DON transport to the fetus has been measured in different species *in vivo* [33-35]. Obviously, in these cases the placenta itself is also exposed to DON which can pose additional health risks. As the placenta facilitates the transport of nutrients and elimination of metabolic waste products, defects in its structure and function result in fetal growth restriction and health problems or even fetal death [36, 37]. The placenta is also responsible for transmission of maternal immune mediators to the fetus [35]. A healthy development of the fetus depends on the regulation of the immunological milieu within the placental environment. An altered immunological status in the placenta underlies many pregnancy complications such as pre-eclampsia and miscarriage [38]. In a previous study, it was demonstrated that DON could disrupt the placental epithelial barrier and induce proinflammatory responses in an *in vitro* model [39]. Although the evidence for adverse developmental effects of DON exposure is clear, very little is known about the effect of DON on the course of pregnancy and the placenta. Therefore, the present study focused on the consequences of chronic exposure to DON-contaminated diet on pregnancy in mice, with a specific focus on changes in the placental structure, as well as immune modulation in the mother and the fetus.

Materials and methods

Animals

Six-week-old, male and female C3H/HeOuJ mice (50 female and 25 male mice), purchased from Charles River Laboratories (Sulzfeld, Germany) were housed at the animal facility of Utrecht University (Utrecht, The Netherlands) at controlled temperature (21 ± 2°C) and humidity (50–55%), with a reversed 12:12 h light/dark cycle (lights on from 7.00 pm till 7.00 am) and with ad libitum access to food and tap water. Animals were kept in makrolon cages (22 cm×16 cm×14 cm, floor area 350 cm2, Technilab- BMI, Someren, the Netherlands), in groups of 2 per cage, with wood-chip bedding (Technilab- BMI, Someren in the Netherlands), and tissues (VWR, the Netherlands) and cardboard shelters were available. Upon arrival, female mice were randomly allocated to the experimental groups (12 mice per group) and all mice were habituated to laboratory conditions for one week prior to the start of the study. The animals received standard control diet (pelleted food, AIN-93G, Ssniff Spezialdiäten, Soest, Germany) and routine care for a week upon arrival in the animal facility before

the start of the experiments. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals of the Utrecht University, and all animal procedures were approved by their Animal Welfare Body under the Ethical license of the national competent authority (Centrale Commissie Dierproeven, CCD).

Diets

To obtain the experimental diets, semi-purified AIN-93G soy protein-based diets were composed and mixed with different concentrations of DON (6.25 or 12.5 mg per kg of diet, FERMENTEK Ltd, Jerusalem, Israel) by Ssniff Spezialdiaten GmbH (Soest, Germany). Maximum permitted levels of DON in different cereals and cereal-based products are set by European Commission and United States to be 1-2 mg/kg of food [40]. However, analyzing wheat, corn and other cereal samples from all over the world revealed a considerable percentage of food product exceeding this safety level [41-43], as DON concentration in several commodities were reported to be as high as 20 mg/kg of food [44]. Moreover, consuming high amounts of cereals and cereal-based products, and combined consumption of a wide variety of DON-contaminated food can lead to a significantly higher exposure levels in specific populations [44].

Experimental design

After 7 days of acclimatization, female mice were fed either AIN-93G (control) or DON-containing diets (6.25 or 12.5 mg DON/kg of diet) for 2 weeks prior to mating. In order to avoid DON exposure in the male mice, all animals received control diet during mating. Males and female mice were mated for 4 days by placing one male mouse in the cage with 2 female mice. Immediately after separating male mice from the females, the female mice (2 per cage) received control/DON diets until the end of experiment. In order to prevent any additional discomfort and stress during gestation period, the animals were weighed only before mating and at the end of experiment to evaluate the weight gain. The first day after starting the mating was considered day 0 of pregnancy.

The female mice were killed by cervical dislocation on day 17 after mating, and samples were collected from mice with live or visible resorbed fetuses (late resorptions) in their uteri. Breeding success was calculated as ratio of pregnant mice with fetus or visible resorption sites in the uterus on day 17 of pregnancy (late resorptions), to all mated female mice. Isolated placentas were either stored in fixation buffer or snap frozen until further processing. Prior to snap freezing, placentas were cut in half to allow RNA extraction and protein extraction from the same placenta. The male mice were available as surplus for other research after mating.

FITC-dextran permeability assay

All animals received FITC-dextran (molecular mass 4 kDa; Sigma-Aldrich) by an oral gavage (300 µl of 50 mg/ml FITC-D solution in PBS), 4 hours before cervical dislocation. The concentration of FITC-dextran in the serum was determined by measuring fluorescence intensity using a spectrofluorometer (FLUOstar Optima; BMG Labtech) set at excitation and emission wavelengths of 485 and 520 nm, respectively, according to the methods described previously [45].

RNA Extraction from placenta and fetal intestine samples and Quantitative qPCR

Placental tissues and fetal intestine were weighed and subsequently homogenized with RNA lysis buffer (provided within RNA isolation kit) with 1:1 (w/v) ratio. The clear supernatants of the homogenates were collected for RNA isolation according to the manufacturer's instructions (SV Total RNA Isolation System, Promega Corporation, Madison, WI, USA). Total RNA content and purity of the samples was measured using the NanoDrop ND-1000 Spectrophotometer (Thermofisher Scientific, Wilmington, DE, USA). cDNA was prepared with use of the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). For qPCR, iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and primers for selected genes (Bio-Rad Laboratories, Hercules, CA, USA) were mixed with the samples. Amplifications were performed according to the manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA quantity was calculated relative to the expression of β-actin reference gene and normalized to the average of control group.

Western blotting of placenta samples

Half of the placenta collected from each pregnant mouse was weighed and homogenized with RIPA Lysis and Extraction Buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) with 1:1 (w/v) ratio. The clear supernatants of the homogenates were used for isolating protein. Total protein concentration was assessed by a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Western blotting was performed according to previously described methods [46]. Primary antibodies for mouse CLDN-3, CLDN-4, ZO-1, OCLD (all from Invitrogen, Carlsbad, CA, USA), and E-cadherin (eBioscience, San Diego, CA, USA), as well as the appropriate secondary antibodies (Dako, Glostrup, Denmark) were used to label the relevant protein bands. Protein bands were visualized by ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA) using ECL reagents mix (Amersham Biosciences, Roosendaal, The Netherlands). The images were analyzed using Image Lab software (version 5.2, 2014, Bio-Rad Laboratories, Hercules, CA, USA). Monoclonal rabbit anti-human β-actin antibody (Cell Signaling, Danvers, MA, USA) was used to evaluate the homogeneity of loading and normalize the optical density of the bands. The protein levels were expressed as the fold change in relation to the average of control group.

Cytokine measurements of placenta and amniotic fluid

Part of the clear supernatants of the placenta homogenates prepared in RIPA buffer, and amniotic fluid samples collected from amniotic sac of fetuses were used to measure the concentration of cytokines interferon gamma (IFN-γ), interleukin (IL)-4, IL-6, IL-10, IL-17, IL-1β and tumor necrosis factor alpha (TNF-α) using ProcartaPlex mouse multiplex immunoassays (Thermofisher Scientific, Wilmington, DE, USA) according to the manufacturer's instructions.

Immunofluorescence staining of placenta

After fixation in 10% formalin, placental samples were embedded in paraffin and 5 µm sections were deparaffinized, and endogenous peroxidase activity was blocked with 0.3% H2O2 (Merck, Darmstadt, Germany) in methanol and rehydrated in a graded ethanol series. Antigen retrieval was performed by incubating the samples in 10 mM citrate buffer (PH 6.0) for 10 min in a microwave. To block non-specific binding sites, 5% goat serum (Dako, Glostrup, Denmark) was used. The sections were incubated with primary antibodies against ZO-1 (1:200, Invitrogen, Carlsbad, CA, USA) or E-cadherin (1:200, eBiosciences, San Diego, CA, USA) followed by Alexa-Fluor conjugated secondary antibodies (1:200, Invitrogen, Carlsbad, CA, USA). Anti-fading mounting medium containing DAPI (ProLong Gold with DAPI, Life Technologies, Thermo Fisher Scientific, Wilmington, DE, USA) was used for nuclear counterstaining and sealing the slides with a coverslip. Localization of the junctional proteins was visualized using the Microscope Leica TCS SP8 X.

DON measurement in fetus samples

Frozen whole fetus samples were weighed, cut into small pieces and homogenized in PBS at 1:1 v/w ratio. The clear supernatant of the homogenates was used to measure DON concentration by Deoxynivalenol ELISA Assay Kit (Eagle biosciences, Amherst, New Hampshire, USA) according to the manufacturer's instructions.

Short chain fatty acid concentration in cecum content of pregnant mice

Cecum content of pregnant mice was homogenized in cold PBS at a ratio of 1:10. Clear supernatants of the homogenates were collected and used for measuring the concentration of acetic, propionic, and butyric acids by gas chromatography, as previously described [47], using 2-ethylbutyric acid as internal standard.

Flowcytometry analysis of spleens and iliac lymph nodes of pregnant mice

Fresh spleen samples collected from pregnant mice were used for isolating splenocytes by methods described previously [48]. Immune cells were labeled using fluorochrome-conjugated monoclonal antibodies against CD4, Tbet (both from BioLegend, San Diego, CA, United States), CD69, CXCR3, CD25, Foxp3, AHR (all from eBiosciences, Thermo Fisher Scientific, San Diego, CA, USA) and T1ST2 (MD Biosciences, St. Paul, MN, USA). A fixable viability dye (eBioscience) was used for assessing cell viability. For detection

of intracellular transcription factors, Foxp3 Staining Buffer Set (eBioscience) was used to permeabilize the cells, according to manufacturer's protocol. Results were collected with BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowLogic software (Inivai Technologies, Mentone, VIC, Australia). The gating strategy for selecting specific T cell populations is shown in supplementary figure 1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8, 2018, GraphPad, La Jolla, CA, USA). Results are expressed as the mean ± SEM and differences among groups were statistically determined using one-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison post hoc test for selected comparisons. For not normally distributed data Kruskal-Wallis test was performed followed by Dunn's multiple comparisons test. Fisher's exact test was used for comparing differences between proportions. Differences were considered significant at $p < 0.05$.

Sample size calculation

Sample size was calculated using G*Power v3.1.9, based on a previous *in vivo* study [31]. The effect size was calculated to be 1.2 based on the expected resorbed implants, and the power was set on 0.9. Correcting alpha based on number of relevant comparisons resulted in the required group size of 6 for each dietary group. Assuming an average breeding success to be 50 (± 5) %, 12-13 female mice were randomly allocated in each dietary group. However, due to the negative effect of DON on breeding success, the number of pregnant mice in groups receiving 12.5 mg DON/kg of diet was lower (n=3), hence the power was recalculated to be 0.8 based on actual number of pregnant mice.

Results

DON exposure during pregnancy significantly affected weight gain and fetal survival.

The effects of feeding mice with DON-contaminated diets on pregnancy outcome is summarized in *table 1*. A concentration of 12.5 mg DON/ kg diet reduced the breeding success (i.e. the number of pregnant mice) and decreased the fetal survival rate. The lowest concentration of DON induced an apparent increase in breeding success; however, the fetal survival rate was lower compared to control group. Overall, the average number of live fetuses per mouse was decreased as fetal survival was reduced in DON-receiving groups.

Numbers of live and visible resorbed fetuses were counted in the uteri of pregnant animals. The gross macroscopic appearance of non-pregnant and pregnant uteri is depicted in figure 1A, where a clear difference can be observed between the uterus with live fetuses and the uterus with late resorptions. The concentration of DON in

fetal homogenates was significantly higher in DON-fed mice, compared to the control group, demonstrating placental transport of DON from maternal to fetal side (figure 1B). The tested concentrations of DON in this study did not significantly affect intestinal permeability of pregnant mice, as the concentration of FITC-dextran in the serum was not significantly different between groups (supplementary figure 2).

Table 1. Effect of deoxynivalenol (DON) exposure during pregnancy on breeding success (%) and fetal survival rate (%). ^{ns} $p > 0.05$, $^{***}p < 0.001$ show significant difference compared to control group, using Fisher's exact test.

	Control	DON 6.25 mg/kg	DON 12.5 mg/kg
Number of mated female mice	12	13	13
Number of pregnant mice (% breeding success)	5 (42%)	8 ^{ns} (61%)	3 ^{ns} (23%)
Number of live fetuses per pregnant mouse	6	4.6	2.6
Number of late resorptions per pregnant mouse	1.5	2.8	5.3
Ratio of live/resorbed fetuses	4	1.6 ^{ns}	0.5***
survival rate (%) = [no. of live fetuses / (no. of live + no. of late resorptions) x100]	(80%)	(61%)	(33%)

DON exposure during pregnancy does not affect SCFA concentrations in the cecum of pregnant mice.

Considering the importance of intestinal bacterial colonization in immune system, concentrations of SCFA in the cecum of pregnant mice were measured as a marker for metabolic activity of gut microbiota (figure 1C-E). No significant effect on concentrations of different SCFAs was observed between different groups, however, a slight decrease in acetic acid concentration was observed in the cecum of mice fed with DON-contaminated diets ($p=0.06$) (figure 1C).

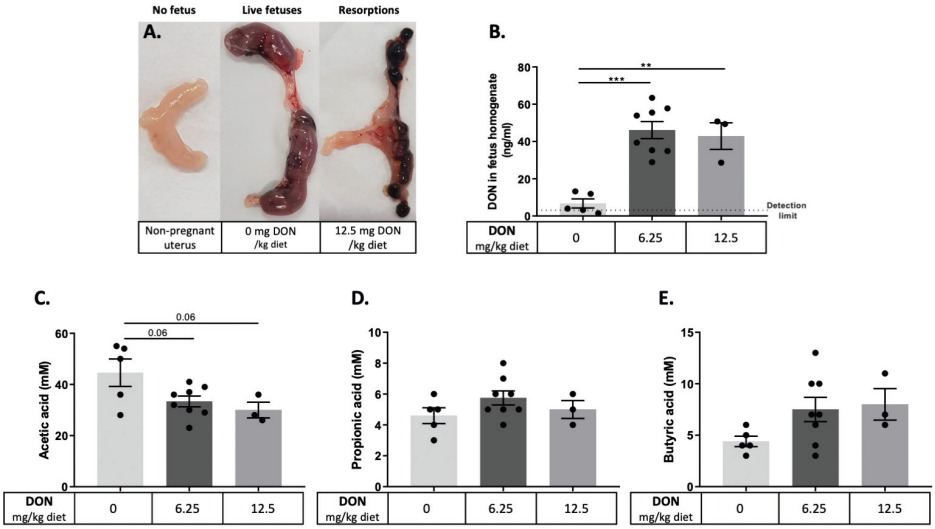

Figure 1. Deoxynivalenol (DON) exposure negatively affects pregnancy outcome and Short-chain fatty acids (SCFA) concentrations in cecum. Pregnant mice were fed either a control or DON-contaminated diets (6.25 or 12.5 mg/ kg of diet). (**A**) Representative picture comparing the uterus from a normal non-pregnant mouse, a healthy pregnant mouse on control diet with live fetuses, and a pregnant mouse receiving 12.5 mg DON/kg diet, with resorbed fetuses; (**B**) the concentration of DON in the whole-fetus homogenates, measured by ELISA. The concentrations of (**C**) acetic acid, (**D**) propionic acid, and (**E**) butyric acid present in the supernatants of homogenates of cecum contents was measured. Data expressed as mean ± SEM, *p<0.05 and ***p<0.001 represent the significant difference.

DON exposure during pregnancy alters Th1/Th2 balance in maternal spleen and lymph nodes.

Results of flowcytometric analysis showed no significant effect of consuming DON-contaminated diets on Th1, Th2, Th17 and regulatory T cells populations in spleen and ILN isolated from pregnant mice (figure 2). Although DON exposure had no significant effect on Th2 in the spleen (figures 2A), a decrease in $T1ST2^{+}$ Th2 cells ($p < 0.05$) could be demonstrated in the ILNs of animals fed with diet containing 12.5 mg DON/kg (figure 2C). Percentages of Th1, Th17 and regulatory T cells in spleen and ILN were not significantly affected by DON exposure (figures 2B, D, E-H). However, a significant reduction in AHR $^{+}CD4^{+}$ T cells was observed in the spleens of DON-exposed animals, compared to the control group (figure 2I).

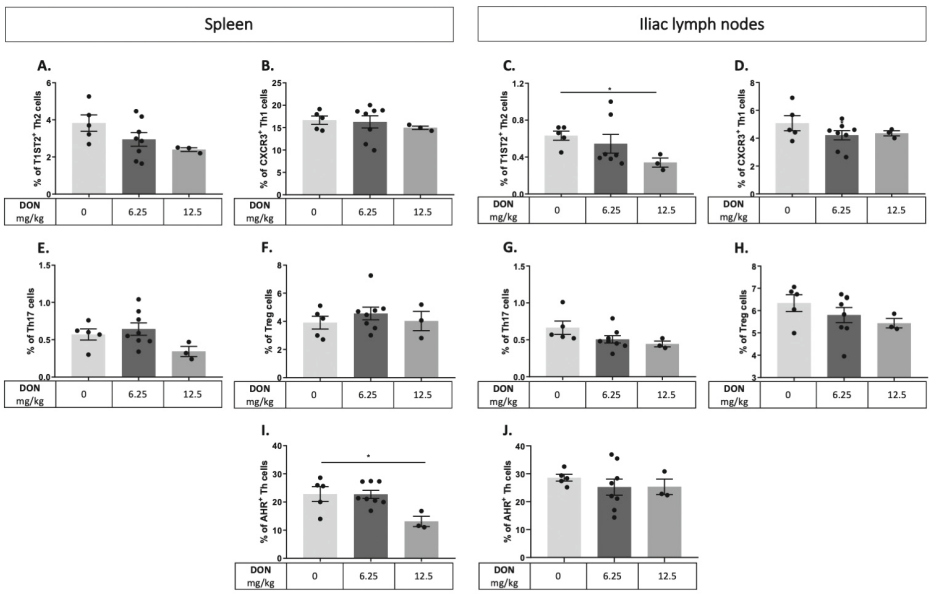

Figure 2. Deoxynivalenol (DON) exposure negatively affects T cell populations in the spleen and iliac lymph nodes of pregnant mice. Pregnant mice were fed either a control or DON-contaminated diets (6.25 and 12.5 mg/ kg of diet). Percentages of $T1ST2^+$ Th2 cells and $CXCR3^+$ Th1 cells were calculated out of live $CD4^+$ T cells in the spleen (**A and B**) and iliac lymph nodes (**C and D**); and percentages of $CCR6^+RoryT^+$ Th17 cells, $CD25^+FopxP3^+$ Treg cells, and aryl hydrocarbon receptor $(AHR)^+$ T cells were calculated out of live $CD4^+$ T cells in the spleen (**E, F and I, respectively**) and iliac lymph nodes (**G, H and J, respectively**), using flowcytometry analysis. Data expressed as mean ± SEM, *p<0.05 represent the significant difference.

DON exposure during pregnancy altered the mRNA and protein expression of junctional proteins in placenta.

The mRNA expression levels (figure 3A-D) and protein concentrations (figure 3E-H) of different junctional proteins were measured in placenta to assess the effect of DON on placental barrier function. Representative protein bands in western blot are shown in supplementary figure 3. Although the mRNA expression level of ZO-1 was not significantly affected (figure 3A), its protein expression was increased in group fed 6.25 mg DON/kg in the diet (figure 3E). DON-contaminated diets significantly downregulated the mRNA expression of E-cadherin, CLDN-3 and 4 (figure 3B, C and D), as well as the protein expression of E-cadherin and CLDN-3 (figures 3F and G), compared to the control diet.

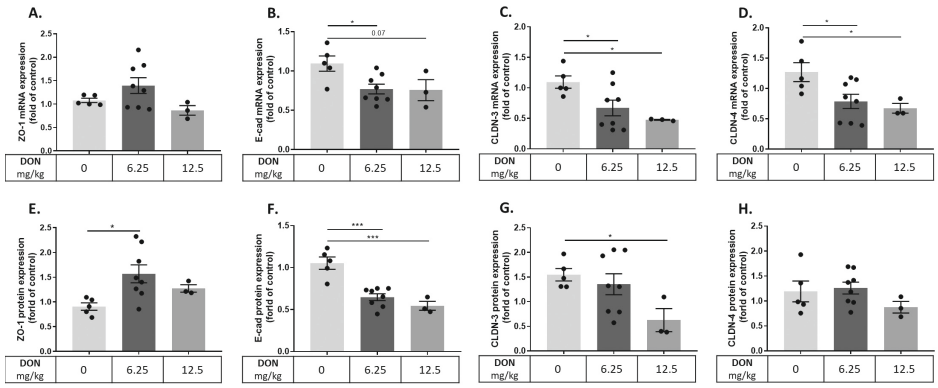

Figure 3. DON exposure during pregnancy alters the mRNA and protein expressions of junctional proteins in the placenta. Pregnant mice were fed either a control or DON-contaminated diets (6.25 and 12.5 mg/ kg of diet). mRNA and protein expressions of (**A, E**) zonula occludens protein-1 (ZO-1), (**B, F**) E-cadherin (E-cad), (**C, G**) claudin (CLDN)-3, (**D**), H CLDN-4 and were measured in placenta homogenates. Data expressed as mean ± SEM of fold of control, normalized to reference gene or protein, *p<0.05 and ***p<0.001 represent the significant difference.

To investigate the cellular localization of junctional proteins, immunofluorescence staining was performed on placenta sections for ZO-1 (a tight-junction protein) and E-cadherin (an adherent-junction protein) (Figure 4). In placental sections from the control group, clear continuous belt-like structures were detectable for both ZO-1 and E-cadherin (Figures 4A/D, and 4G/J, respectively). However, in placenta sections of DON-fed mice, especially the higher DON dosage (12.5 mg DON/kg), these belt-like structures for ZO-1 and E-cadherin were not detected (figures 4C/F and I/L, respectively).

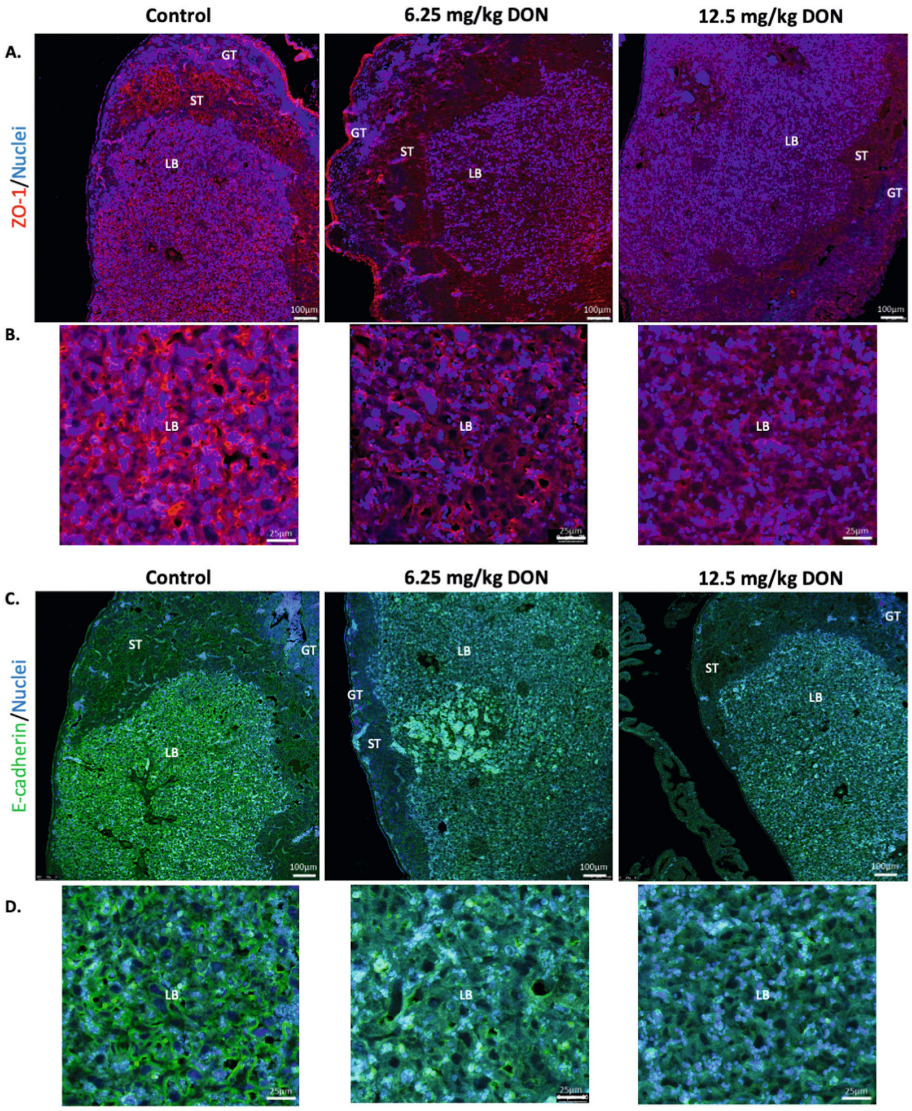

Figure 4. Deoxynivalenol (DON) exposure during pregnancy alters the cellular localization of zonula occludens protein-1 (ZO-1) protein and E-cadherin in the placenta. Pregnant mice were fed either a control or DON-contaminated diets (6.25 mg/kg and 12,5 mg/kg of diet). The placental sections were visualized by immunofluorescence staining for: **A-B**) ZO-1 (in red) and nuclei (in blue), and **C-D**) E-cadherin (in green) and nuclei (in blue). Scale bars represent 100µm (**A, C**), and 25µm (**B, D**). LB: labyrinth zone, ST: spongiotrophoblasts, GT: trophoblast giant cells.

DON exposure during pregnancy alters the mRNA expression levels of immune markers in placenta.

The mRNA expression levels of different immune markers were assessed in placental homogenates of control and DON-fed mice to determine immunological changes in the placenta. Feeding the mice DON-contaminated diets induced downregulation of CD80 and CD86 mRNA in placenta (figure 5A and B), and upregulation of AHR mRNA (figure 5C), and, compared to control group, while had no effect on placental Tbet, FoxP3 and RorγT expressions (figure 5D-F).

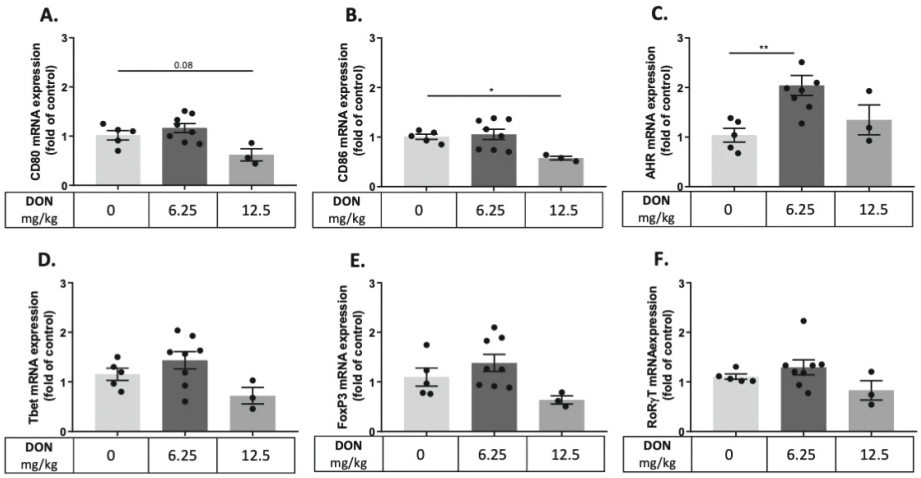

Figure 5. DON exposure during pregnancy alters the mRNA expression of immune cell markers in the placenta. Pregnant mice were fed either a control or DON-contaminated diets (6.25 and 12.5 mg/ kg of diet). mRNA expression of (**A**) CD80, (**B**) CD86, (**C**) aryl hydrocarbon receptor (AHR), (**D**) Tbet (Th1-cell marker), (**E**) FoxP3 (regulatory T-cell marker), and (**F**) RoRγT (Th17-cell marker) were measured in placenta homogenates. Data expressed as mean ± SEM of fold of control, normalized to housekeeping gene, *p<0.05 and **p<0.01 represent the significant difference.

DON exposure during pregnancy alters cytokine levels in placenta and amniotic fluid.

The concentration of different cytokines in the placenta homogenates and amniotic fluid is depicted in figure 6. Presence of DON in the diet, both concentrations of 6.25 mg and 12.5 mg/ kg, significantly increased the concentration of IFN-γ, IL-6 and IL-4 in placental samples (figures 6A, B and C), while no effect on IFN-γ and IL-6 concentrations in amniotic fluid were observed (figures 6D and E). Although not significantly different (p=0.07), the concentration of IL-10 was decreased in the amniotic fluid of mice fed the 12.5 mg/kg DON diet (figure 6F). Concentrations of IL-1β, TNF-α and IL-10 in placenta, and IL-1β, TNF-α and IL-4 in amniotic fluid were below the detection limits of the ELISA kit used in this experiment (data not shown).

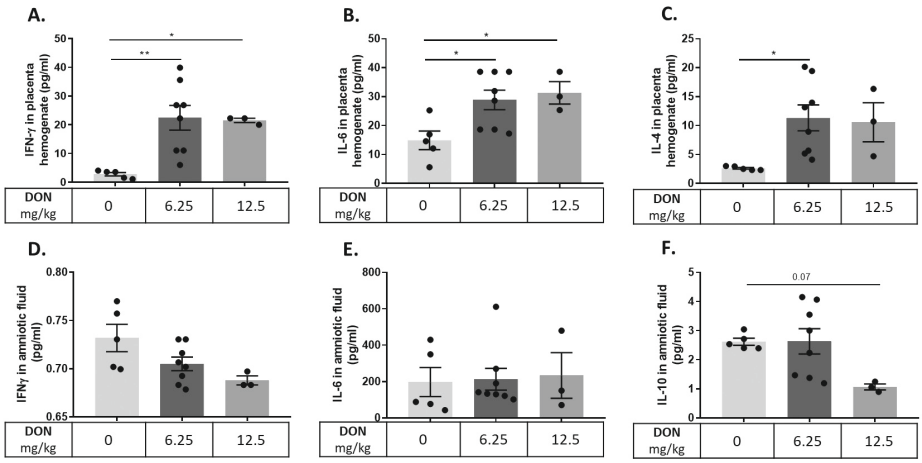

Figure 6. DON exposure during pregnancy alters cytokine production in placental homogenates and amniotic fluid. Pregnant mice were fed either a control or DON-contaminated diets (6.25 and 12.5 mg/ kg of diet). Concentrations of (**A, D**) Interferon (IFN)-γ, (**B, E**) interleukin-6 (IL-6), (**C**) IL-4, and (**F**) IL-10 were measured in clear supernatant of placenta homogenates and amniotic fluid, respectively. Data expressed as mean ± SEM, *p<0.05 and ***p<0.001 represent the significant difference.

DON exposure during pregnancy does not affect junctional protein expression in the fetal intestine.

Considering the adverse effect of direct exposure to DON on intestinal barrier integrity through changing the expression and localization of tight junction proteins [45], gene expression of junctional proteins in intestine samples of the fetuses were measured. However, no significant difference in mRNA expression of ZO-1, OCLD, E-cadherin and CLDN proteins was observed between different groups (supplementary figure 4).

Discussion and conclusion

Pregnant mothers are exposed to a wide variety of environmental toxins and chemicals, which can adversely affect the process of pregnancy and fetal development, either by modulating the maternal parameters such as immune responses, or by passing through the placenta and reaching to the fetus. As the placenta is the most critical organ in maintaining a healthy pregnancy, understanding the effects of xenobiotics on placental structure and function should provide the basis for understanding and predicting aspects of developmental toxicity [49]. DON is a highly prevalent food contaminant known to induce embryotoxicity and reproductive toxicity [50]. In order to gain insight into the possible mechanisms involved in developmental toxicity of DON, the present study investigated the effects of dietary exposure to DON during pregnancy on both maternal and fetal side, as well as the placental structure, focusing on immune and barrier function.

DON exposure negatively affects pregnancy and modulates the maternal immune organs and intestinal microbial activity

Feeding pregnant mice with DON-contaminated diets adversely affected fetal survival, demonstrated by increased fetal mortality and decreases in breeding success. The ratio of live to resorbed fetuses (late resorptions) was considerably decreased with all tested concentration of DON, while the effect of DON on breeding success (*i.e.* the number of pregnancies) was more prominent with 12.5 mg DON /kg of diet. Similar observations were reported in rats fed with 3-5 mg DON /kg of body weight throughout the gestation period, leading to reduction in weight gain during pregnancy and an increase in number of totally resorbed litters [29, 51]. In the current study resorbed implantation in early stages of pregnancy, which did not leave a macroscopic lesion in the uterus on gestation day 17, were not included. However, considering that the female mice received DON-contaminated diets even before mating and throughout the pregnancy starting immediately after mating, there is a high chance of early resorptions during first days of implantation. Thus, further experiments are needed to get a more accurate evaluation of the impact of DON on both early and late fetal resorptions. Furthermore, DON is shown to inhibit the oocyte maturation, and reduce oocyte quality [52, 53]. Considering that in this study female mice received DON-contaminated diets before mating, the reduction in breeding success and reproductive performance could be partially explained by adverse effects of DON on oocytes and induction of pre-implantation losses. Further investigations are required to compare the pre- and post-implantation losses and determine the critical window for DON-induced reproductive toxicity.

SCFAs, as products of intestinal bacterial metabolism, are involved in G-protein receptor signaling pathways which are recognized as a molecular link between diet, microflora and immune response [54]. Intestinal SCFAs levels play a critical role during pregnancy, as

changes in the intestinal microbial content and metabolism during pregnancy contribute to the occurrence of metabolic changes in the mothers and increase inflammatory markers [55]. In the present study, a tended reduction in concentration of acetic acid in cecum was observed in pregnant mice receiving DON-contaminated diets. Pregnancy increases the concentration of SCFAs in the maternal serum and intestinal lumen [56], and acetic acid is known as the dominant SCFA in both pregnant women and their infants [57]. Gestational serum acetate concentrations, which are most likely mirrored by the acetate production in the intestine, are positively associated with gestational weight gain, maternal adiponectin and leptin levels, as well as neonatal growth [58]. The DON-induced reduction in acetate concentration in cecum may contribute to the adverse health effects in the mother and the neonates.

Changes in the maternal immune system may play a crucial role in reduced fetal survival. In the current study, flowcytometric analysis of maternal spleen and iliac lymph nodes revealed a slight decrease in Th2 cell populations in ILN of mice fed with 12.5 mg/kg of DON, thereby potentially shifting the Th1/Th2 balance towards Th1. During a healthy pregnancy, the maternal immune system shifts towards a more tolerogenic state by downregulating Th1-mediated immune responses and increasing production of regulatory and Th2-mediated cytokines, in order to prevent rejection of the semi-allogeneic fetus [59]. Therefore, an optimal balance of Th1/Th2/Treg immunity, with a slight shift to Th2-type immunity, is required for the maintenance of a successful pregnancy. Although results of the present study showed no significant change in regulatory T cell population in maternal immune organs, a shift in Th1/Th2 balance may have contributed to the increased resorption in DON-exposed mice.

DON exposure alters placental structure and immunity

The placenta, being the only connection between the fetus and the mother, is the most critical organ for maintaining a healthy pregnancy. The main structural components of the placenta are the extensive villous branches forming layers of labyrinth zone, the spongiotrophoblasts zone, and the outer trophoblast giant cells. The maternal blood passes through the spongiotrophoblast via large arterial sinuses, and eventually enters into the labyrinth space where the material exchange between the mother and fetus takes place [60]. In the present study, significant changes in expression and cellular localization of junctional proteins in the placenta of DON-exposed mice were observed, demonstrated by downregulation of protein and mRNA expression levels of E-cadherin and claudin proteins and structural changes in the belt-like intercellular networks of ZO-1 and E-cadherin, which was more obvious in labyrinth zone. The placenta forms a selective barrier at the maternal-fetal interface, mediating transfer of nutrients and metabolic waste products [61], and preventing transmission of pathogens, chemicals and toxins [62]. Therefore, disruption of the integrity of placental barrier can lead to imbalanced maternal-fetal transportation of nutrients and hazardous chemicals. The present study is the first to report effect of DON on placental barrier markers in a

murine model. In line with these observations, an earlier *in vitro* study in our group showed direct exposure to DON induces significant changes in expression level and localization of different junctional proteins in placental epithelial barrier [63].

In addition to the barrier function and critical role in maternal-fetal transportation, the placenta is able to produce and respond to a great diversity of inflammatory stimuli [64], thus a regulated immune response and cytokine production in placenta is required for maintaining a healthy pregnancy. An increase in concentrations of IL-4, IL-6 and IFN-γ was observed in the placenta of DON-exposed mice. Excessive and imbalanced activation of T and NK cells in placenta leads to pro-inflammatory cytokine production, which in turn can cause pregnancy loss through induction of placental infarction and thrombosis [65]. Moreover, a significant reduction in IL-10 concentration was observed in the amniotic fluid of DON exposure animals. Cytokines at the maternal-fetal interface, such as placenta and amniotic fluid, play a crucial role in successful pregnancy. Elevated production of inflammatory cytokines such as IFN-γ and IL-6 can exert detrimental effects in the placenta and induce abortion [66, 67], whereas the regulatory cytokine IL-10 is beneficial for maintaining a healthy pregnancy [68] and preventing preterm labor associated with inflammation or infection [69]. In an *in vivo* study in pregnant mice, beneficial properties of dietary supplementation with proline on enhancing fetal survival and placental development were linked to lower concentration of inflammatory cytokines such as IFN-γ and IL-17, and higher IL-10 production in placenta and amniotic fluid [70]. Furthermore, excessive levels of maternal IL-6 can be transferred to the fetal side [71], which can lead to long-term adverse effects on the offspring, including insulin resistance, elevated stress response as well as hypertension in adulthood [72, 73]. Altogether, changes in cytokine production in the placenta of DON-exposed mice reflects an imbalanced immune response in the placenta, which may adversely impact fetal development and pregnancy.

Moreover, the expression of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor responsible for activating the metabolism of environmental and food-associated xenobiotics [74, 75], was measured in the placenta and maternal immune organs. AHR has a crucial role in differentiation and proliferation of regulatory T cells [76], as well as the fetal survival during pregnancy [77, 78]. Therefore, a tightly regulated expression of AHR is essential in maintaining a healthy pregnancy and fetal development [79]. To our knowledge, the present study is the first to investigate the effect of DON exposure on the expression of AHR during pregnancy. Interestingly, placental mRNA expression of AHR was increased in mice receiving the diet containing 6.25 mg DON/kg, whereas flowcytometric analysis of maternal immune organs revealed a reduction in AHR^+ cells in spleens of pregnant mice fed the diet with 12.5 mg DON /kg. It is not yet clear what these outcomes may signify. However, DON-induced alterations in AHR expression in maternal organs and maternal-fetal interface observed in the current study may have contributed to the observed differences in pregnancy

outcome, as an increase in AHR expression and activation in the placenta is associated with impaired trophoblast cell proliferation and migration, which can consequently lead to miscarriage and abortion [80, 81]. Additionally, it is very well known that many immunotoxic compounds induce their effects at least in part via AHR receptors in thymus, liver and even skin. Nevertheless, further experiments with higher sample size are required to fully understand the impact of DON on AHR and its consequences in pregnancy.

Direct DON exposure through placenta may contribute to the detrimental effects on the fetus

Detection of DON in the fetuses of mice fed DON-contaminated diets is consistent with the previous observations, confirming that DON can be transferred across the placenta [82]. Direct exposure of the fetus to DON may, in part, account for the developmental toxicity of DON during pregnancy, such as induction of fetal skeletal deformities [30, 31]. Although DON is known to induce intestinal barrier disruption upon direct exposure, in the present study no significant effect on mRNA expression of fetal intestinal junctional proteins were observed. A possible explanation for this observation could be the low concentration of DON at the fetal side. Similarly, feeding pregnant sows with diets containing 4.42 mg DON/kg diet induced no macroscopic lesions or detectable pathomorphological changes in fetal liver, spleen, lymph nodes, thymus, gut, and bone marrow [83]. However, in another study intrauterine administration of DON in pregnant sows induced a significant impact on T cell populations in piglets after birth, indicated by lower percentages of regulatory and T helper cells in the blood samples and reduced capacity to produce pro-inflammatory cytokines [84]. Considering the well-stablished immunotoxic properties of DON [85], placental transfer of DON can impose adverse immunologic effects on the offspring, as most important immune maturation events occur in early-life, interference with the developing immune system can lead to long-term immune dysfunction in adulthood. Indeed, in another recently published paper we were able to demonstrate that the offspring of mice exposed to similar levels of DON during pregnancy and lactation were more susceptible to develop allergic responses in a murine model for ovalbumin-induced food allergy, and vaccination responses to the Influenza vaccine were lower in these mice [86].

Concluding remarks

Dietary DON exposure during pregnancy has a negative effect on breeding success and fetal survival. Increased early death in DON-exposed mice could be related to the DON-induced imbalanced immune responses in maternal organs, as well as the modifications in the placental barrier and immune function. Moreover, DON reaches the fetus and can induce direct toxicity on the developing fetus. Herein, this study is the first demonstrating changes in placental barrier and immune markers. Overall, results of the present study stress the importance of maternal diet during pregnancy and awareness of the potential harmful effects of commonly present food contaminants

on the course of pregnancy and fetal development. Exposure to the toxic compounds such as DON during early stages of development can potentially lead to long-term and persisting adverse health effects in the later stages of life.

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Author contributions
The author's responsibilities were as follow: BL, SB, AH, NST: designed the research; BL, SB, AH, JG, GF: supervised data interpretation; NST, ST: conducted the in vivo experiment; NST: analyzed data and wrote the paper; All authors provided critical intellectual input for data interpretation, read and approved the final manuscript.

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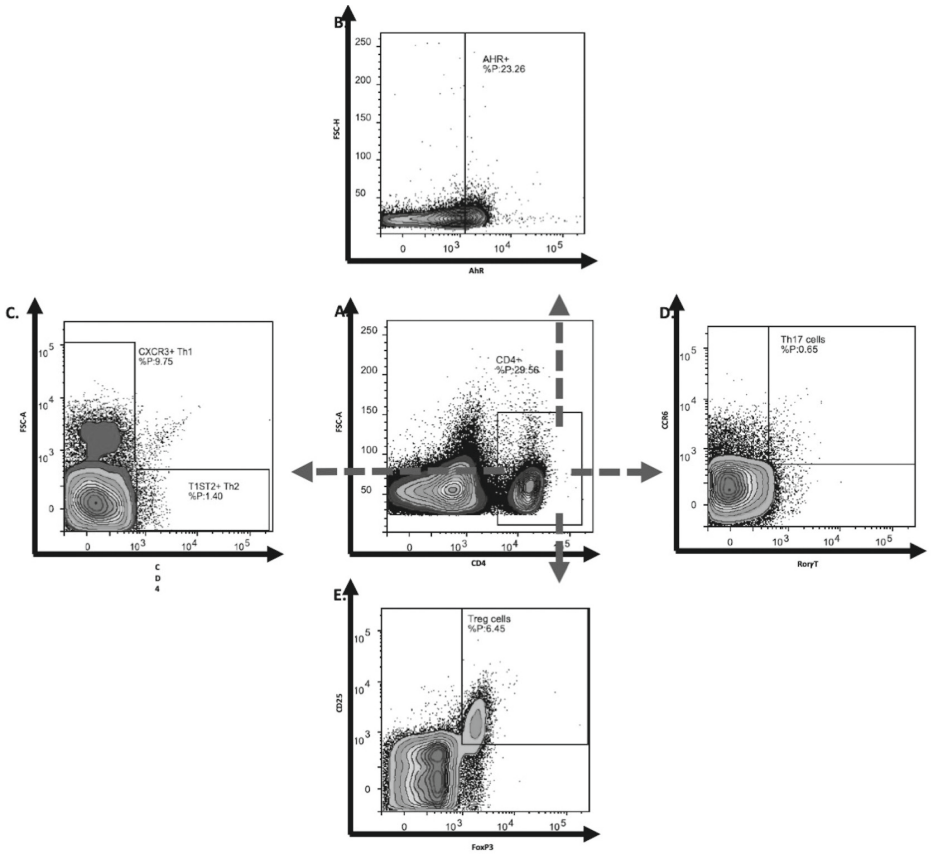
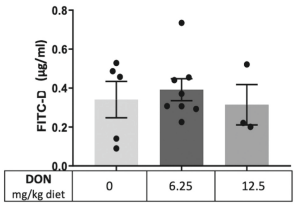
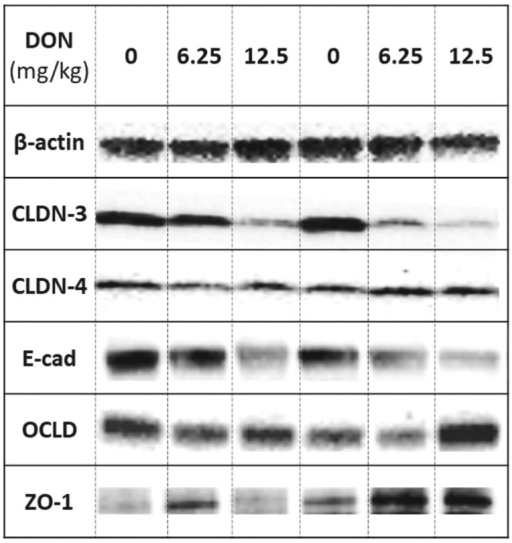
Supplementary content



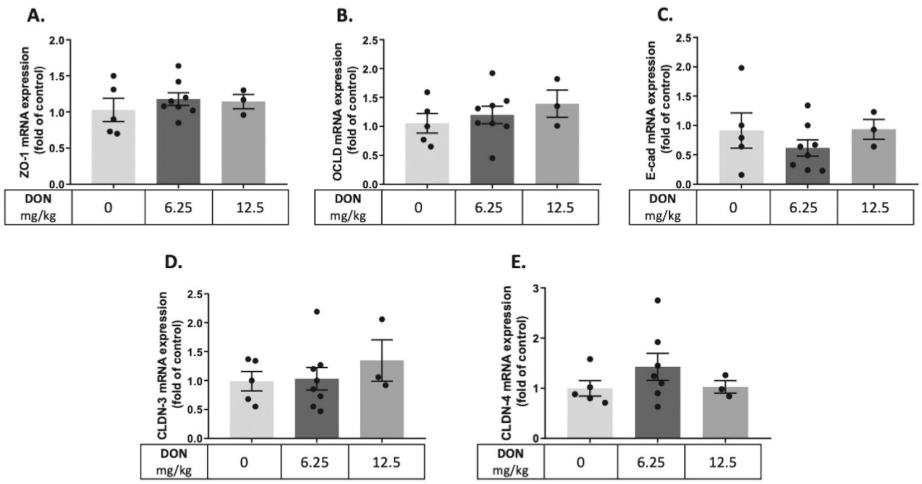
Supplementary figure 1. Representative dot-plots, demonstrating the gating strategy for selecting (**A**) $CD4^+$ T cells from live cells; and (**B**) AHR^+ T cells, (**C**) $CXCR3^+$ T helper 1 and $T1ST2^+$ T helper 2 cells, (**D**) $RoryT^+CCR6^+$ T helper 17 cells, and (**E**) $CD25^+FoxP3^+$ regulatory T cells, out of $CD4^+$ cells, in spleen and iliac lymph node, using flowcytometric analysis.



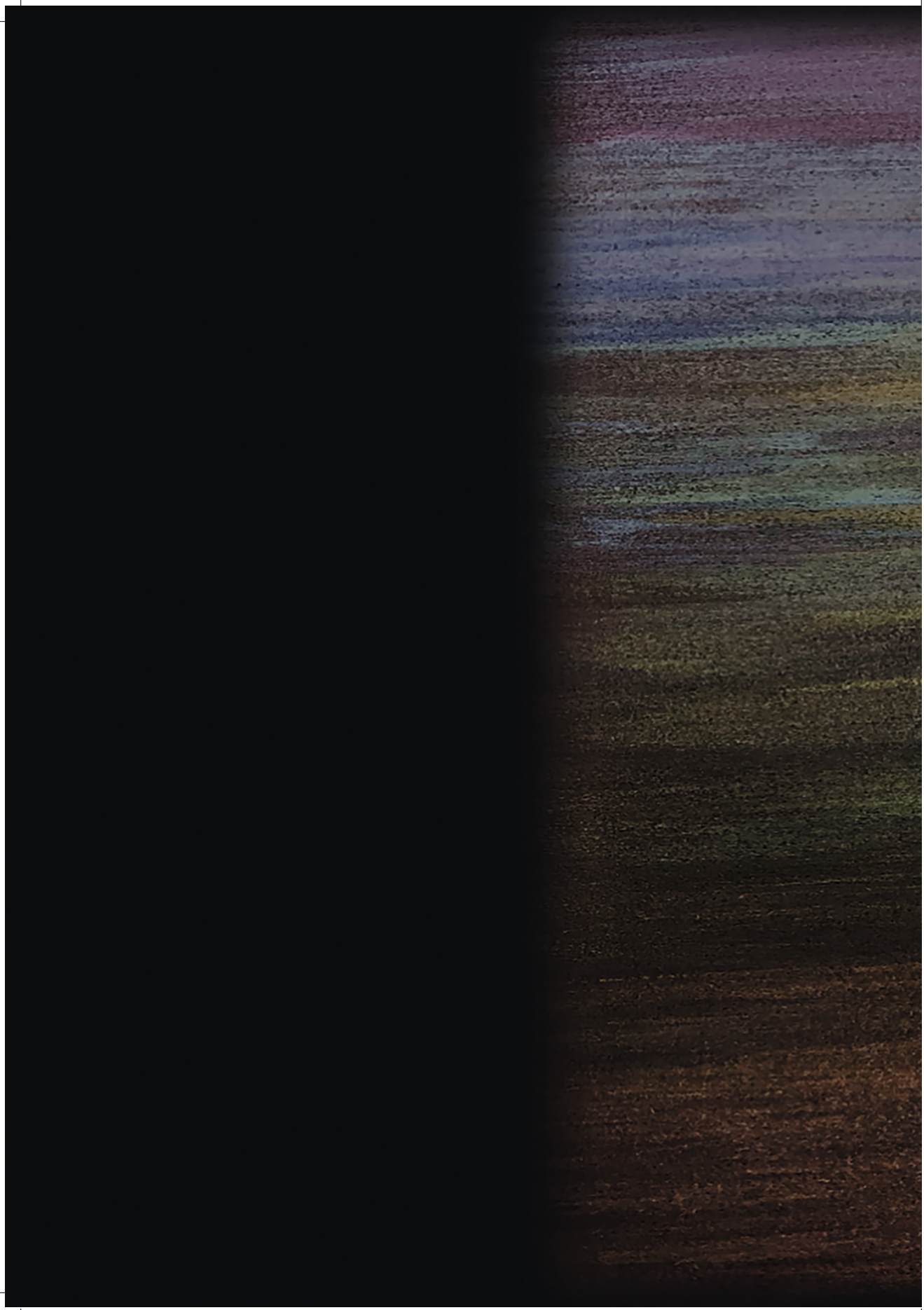
Supplementary figure 2. Concentration of FITC-dextran (FITC-D, 4-kDa) in the serum samples of pregnant mice, fed either a control or DON-contaminated diets (6.25 or 12.5 mg/ kg of diet) throughout the pregnancy. The concentration of FITC-D was determined on day 17 of gestation, 4 h after oral gavage.



Supplementary figure 3. Representative protein bands for junctional Proteins in the placenta collected from pregnant mice receiving deoxynivalenol (DON)-contaminate diets (0 – 6.25 – 12.5 mg/kg of diet) throughout the pregnancy. Occludin (OCLD), zonula occludens protein-1 (ZO-1), claudin (CLDN)-3 and 4, E-cadherin (E-cad).



Supplementary figure 4. Effect of maternal deoxynivalenol (DON) exposure on mRNA expression of junctional proteins in fetal intestine. Relative mRNA expression (fold of control, normalized to β-actin) of (**A**) zonula occludens-1 (ZO-1), (**B**) occludin (OCLD), (**C**) E-cadherin (E-cad), (**D**) claudin-3 (CLDN-3) and (**E**) CLDN-4 in the placenta of mice fed ether control or DON-contaminated diets (0, 6.25 and 12.5 mg/kg) during entire period of pregnancy. Data are presented as mean±SEM



Part 3

Long-Term Effects on Immune Development

Chapter 6

Exposure to deoxynivalenol during pregnancy and lactation enhances food allergy and reduces vaccine responsiveness in the offspring in a mouse model

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Abstract

Deoxynivalenol (DON), a highly prevalent contaminant of grain-based products, is known to induce reproductive- and immunotoxicities. Considering the importance of immune development in early-life, the present study investigated the effects of perinatal DON exposure on allergy development and vaccine responsiveness in the offspring. Pregnant mice received control or DON-contaminated diets (12.5 mg/kg diet) during pregnancy and lactation. After weaning, female offspring were sensitized to ovalbumin (OVA) by oral administration of OVA with cholera toxin (CT). Male offspring were injected with Influvac vaccine. OVA-specific acute allergic skin response (ASR) in females and vaccine-specific delayed-type hypersensitivity (DTH) in males were measured upon intradermal antigen challenge. Immune cell populations in spleen and antigen-specific plasma immunoglobulins were analyzed. In female CT+OVA-sensitized offspring of DON-exposed mothers ASR and OVA-specific plasma immunoglobulins were significantly higher, compared to the female offspring of control mothers. In vaccinated male offspring of DON-exposed mothers DTH and vaccine-specific antibody levels were significantly lower, compared to the male offspring of control mothers. In both models a significant reduction in regulatory T cells, $Tbet^{+}$ Th1 cells and Th1-related cytokine production of the offspring of DON-exposed mothers was observed. In conclusion, early-life dietary exposure to DON can adversely influence immune development in the offspring. Consequently, the immune system of the offspring may be skewed towards an imbalanced state, resulting in an increased allergic immune response to food allergens and a decreased immune response to vaccination against influenza virus in these models.

Introduction

Pregnancy and lactation represent crucial periods in the development of the newborn's immune system [1]. A wide range of contaminants present in maternal food and environment during these periods can interfere with the process of immune programming, leading to long-term or permanent changes in the offspring [1, 2]. The most critical immune maturational events occur during the early-life stages [3, 4]. Therefore, any immune disturbance in this early period can result in altered immune function and can even have significant long-term consequences for the offspring [5, 6]. A strong connection between developmental immunotoxicity and the elevated risk for immune related disorders has been suggested. Diseases such as childhood asthma and allergies [7], chronic otitis media, type-1 diabetes, childhood leukemia and pediatric celiac disease are all related to disturbed and imbalanced immune capacity during early stages of immune development [8]. During pregnancy, the maternal immune system shifts towards a distinct and more tolerogenic state by down regulating Th1-mediated immune responses and increasing production of Th2-mediated cytokines, in order to prevent Th1-driven rejection of the semi-allogeneic fetus [9]. Similarly, the immune system of the newborn after birth is unbalanced and skewed toward Th2 responses [7]. Moreover, the infant is born with an immature (though functional) immune system which will naturally mature after exposure to different antigens and infections during the first months and years of life. Any prenatal or neonatal environmental factors that interfere with the immune programming in early-life, can impose a risk for allergy development and diminished host resistance to disease.

Mycotoxins are among the most important and highly prevalent nutritional contaminants with well-established immunotoxic properties [10]. They are naturally produced as secondary metabolites of different fungal species, which can contaminate a wide range of agricultural products, especially cereal and grain-based food [11]. As a result of the high prevalence of fungal contamination in the food chain, mycotoxin exposure is almost inevitable. Epidemiological studies from different geographical regions have shown that pregnant women and newborns are highly exposed to different mycotoxins [2]. Deoxynivalenol (DON), a trichothecene mycotoxin produced by different Fusarium fungi species, is one of the most prevalent mycotoxins occurring in human food [12]. DON exhibits intestinal, neurological, reproductive and immunotoxicity [13]. Considerable concentrations of DON were detected in urine samples of pregnant women in different geographical regions, some exceeding the proposed maximum tolerable daily intake (TDI; 1 µg/kg of body weight per day) [14, 15]. DON can pass through the placenta and reach the fetus during pregnancy [16-18], and is transferred into the milk during lactation [18, 19], which signifies the importance of exploring the potential adverse effects of DON exposure in newborns. Direct exposure to DON induces immunotoxicity, even with very low doses [20]. Depending on the concentration and duration of exposure, DON can induce both immunosuppressive and immunostimulatory effects

[21]. Immunotoxicity of DON might be induced through oxidative stress and DNA damage [22], and inhibition of lymphocyte proliferation [23]. Previously, it was shown that sensitizing mice by intragastric gavage of whey proteins in combination with DON (100 µg per mouse), enhances allergic reactions to whey proteins, possibly by disturbing the integrity of the intestinal epithelial barrier as an adjuvant, and inducing cell stress, resulting in the initiation of Th2 responses and allergic reactions [24]. Moreover, a compromised resistance to enteric and pulmonary reovirus infections was reported after DON exposure in mice models [25, 26], possibly due to suppression of type-1 IFN-mediated responses.

Although immunomodulatory effects of direct exposure to DON are extensively studied [10, 20-22], information about the immunotoxicity of exposure to DON during pregnancy is scarce [27]. Intravenous administration of DON in pregnant pigs at the end of gestation was shown to alter mRNA expression of IFN-γ, IL-17, IL-2, and TNF-α, in the blood leukocytes of the piglets, and induce significant decrease in the population of regulatory T cells, and an increase in cytotoxic and γδ T cells in blood samples of these piglets, 1-3 weeks after birth [27]. These changes indicate a disturbed and unbalanced immune system in piglets of DON-exposed mothers after birth. To our knowledge, there are no data available on the outcome of early life DON exposure during pregnancy and lactation on the development of immune-related disease or vaccination responses in the later stages of life. Considering the potential immunotoxicity of DON and its effect on Th1/Th2 immune responses, in the current study the effect of maternal exposure to DON during pregnancy and lactation on the immune maturation and Th1/Th2 balance in the offspring was investigated using the ovalbumin (OVA)-induced food allergy model to examine the allergic response, and the Influenza-vaccination model to assess the vaccination response.

Materials and methods

Animals and diets

Eight-week-old female and male C3H/HeOuJ mice were purchased from Charles River Laboratories and housed in the animal facility of Utrecht University at controlled temperature (21 ± 2°C) and humidity (50–55%), with a reversed 12:12 hours light/dark cycle (lights on from 7.00 pm till 7.00 am) and with *ad libitum* access to food and tap water. All experimental procedures were carried out during the dark cycle and all repeated measurements were performed at the same time block of the day. Animals were kept in makrolon cages (22 cm×16 cm×14 cm, floor area 350 cm2, Technilab-BMI, Someren, the Netherlands) with wood-chip bedding (Technilab- BMI, Someren in the Netherlands), and tissues (VWR, the Netherlands) and shelters were available as cage enrichments. The animals received standard diets (pelleted food, AIN-93G, Ssniff Spezialdiäten, Soest, Germany) and routine care for a week upon arrival in the animal facility before the start of breeding. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the Utrecht University, and all animal procedures related to the purpose of the research were approved under license of the national competent authority, securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Semi-purified AIN-93G soy protein-based diet was composed (the details of the diet content have been previously described [28])and mixed by DON (FERMENTEK Ltd, Jerusalem, Israel) at concentration of 12.5 mg per kg of diet, by Ssniff Spezialdiäten GmbH (Soest, Germany).

Study design

A schematic overview of the study design is shown in figure 1. After an acclimatization period, animals were weighed and mated by putting one male mouse together with 2 female mice in the home-cage of the females, for 4 days. After mating, the male mouse was removed from the cage and female mice were randomly assigned to a dietary group, in a way that the average weight of the animals in each dietary group was not significantly different. Control group received standard AIN-93G diet, and DON group received AIN-93G diet containing 12.5 mg DON (per kg of diet) throughout the entire period of pregnancy and the first 2 weeks of the lactation period. Results of a preliminary experiment in our group showed that concentration of 12.5 mg/kg of DON is not inducing acute toxicity, or significant weight loss in mice (unpublished data). Although the concentration of DON used in this study is above the maximum permitted levels of DON in different cereals and cereal-based products (1-2 mg/kg of food) [29], numerous studies have reported a considerable percentage of food products exceeding this safety level [30-32]; DON concentration in several commodities were reported to be as high as 20 mg/kg of food [33]. Moreover, consuming high amounts

of cereals and cereal-based products, and combined consumption of a wide variety of DON-contaminated food can lead to a significantly higher exposure levels in specific populations [33]. Thus, the concentration of DON used in this study can be considered relevant to the human exposure in the areas with high DON contamination.

In order to prevent any direct digestion of DON by the offspring, all lactating mothers received control diet during the last week of lactation. Breeding female mice were weighed on day 0, before starting DON-diet, and at end of lactation period, to evaluate the weight gain in these animals. After weaning, all offspring were put on control diet. After separating the males from the females, the offspring from either control dams or DON-treated dams were briefly grouped together, after which the offspring were randomly allocated to the different treatment groups. Both female and male offspring were housed as 4 and 3 animals per cage, respectively. Female offspring were used in ovalbumin (OVA) food allergy model, and male offspring were used in vaccination model. Details of the sample size calculation and total number of animals in each group is explain in the statistical analysis.

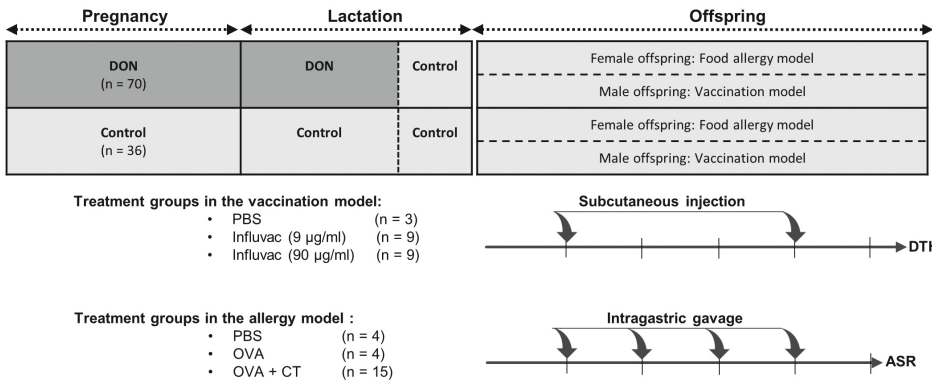

Figure 1. Schematic overview of study design. Female mice received either control (AIN93) or Deoxynivalenol-contaminated (DON) diets during pregnancy and lactation period, until a week before weaning. The offspring received control diet right after weaning until the end of the experiment and were used in either 1) vaccination model (male offspring), receiving 2 injections of phosphate-buffered saline (PBS) or Influvac; or 2) food allergy model (female offspring), receiving intragastric gavage of PBS, ovalbumin (OVA) or OVA with cholera toxin (CT). The number of animals in each group is indicated as n. At the end of the experiment, the animals in vaccination model underwent delayed-type hypersensitivity (DTH) assay, and the animals in food allergy model underwent acute allergic skin response (ASR) assay.

A. Food allergy model

One week after weaning, 4-week-old female offspring were sensitized to OVA according to the previously established OVA-specific food allergy model [34]. Briefly, the animals received oral gavage of either phosphate-buffered saline (PBS; control group), OVA (OVA-sensitized group; 20 mg in 500 µL PBS; grade V; Sigma-Aldrich, Zwijndrecht, The Netherlands) or OVA together with cholera toxin (CT, 10 µg in 500 µL PBS) as an adjuvant (OVA+CT-sensitized allergy group), once a week for 4 weeks. One week after the last sensitization, acute allergic skin responses (ASR) were measured by intradermal challenge, as described below. Six hours after the intradermal challenge, 9-week-old mice were challenged orally with OVA (100 mg in 500 µL) and 15 hours after the oral challenge, blood samples were collected by orbital extraction under isoflurane-induced inhalation anesthesia, followed by cervical dislocation. Thereafter, tissue samples were collected for *ex vivo* analyses.

A.1. Acute allergic skin response

Acute allergic skin response (ASR) was measured by intradermal injection of OVA (1 pg in 25 µL PBS) into the pinna of left ear and 25 µL PBS into the pinna of right ear, under isoflurane-induced inhalation anesthesia. Changes in ear thickness, as a readout for Th2-mediated allergic reaction, was measured 1 hour after injection by using a digital micrometer (Mitutoyo Digimatic 293561, Veenendaal, The Netherlands). Based on earlier studies, ear swelling reaches to an optimum level 1 hour after the antigen challenge [35]. The ASR was calculated by following formula:

ASR = (right ear (thickness at 1h - thickness at 0h)) - (left ear (thickness at 1h - thickness at 0h)

A.2. Anaphylactic shock score and body temperature.

To evaluate the allergic response in the ovalbumin-sensitized offspring, anaphylactic reactions, such as scratching around nose or swollen eyes (table 1) and drop in body temperature as clinical shock symptoms were determined 45 min after intradermal challenge. To establish the severity of the shock, a previously established anaphylactic scoring table was used [34]. Animals were closely monitored after intradermal challenge and body temperature was detected using a rectal thermometer (Terumo, Leuven, Belgium). In case of severe shock symptoms and drop in body temperature, animals were placed on heating pads.

Table 1. The list of anaphylactic symptoms used as the scoring criteria in food allergy model.

Score	Anaphylactic shock symptoms
0	no symptoms
1	scratching around nose and/or mouth
2	swollen eyes and/or mouth, piloerection, reduced mobility, increased breath frequency
3	shortness of breath and/or increased breath frequency, bluish color around mouth and tail, further reduced/painful mobility
4	no mobility following stimulation, convulsions

A.3. Serum concentration of specific antibodies and mouse mast cell protease-1 (mMCP-1)

Blood samples collected from female offspring were centrifuged (12000 RCF for 10 min) to collect the serum and were stored at −20°C until analysis. OVA-specific IgG1, IgG2a and IgE were measured in serum samples of female offspring using enzyme-linked immunosorbent assay (ELISA), as described previously [36]. Concentrations in test sera were calculated in arbitrary units (AU), relative to the standard curve of pooled plasma. Additionally, concentration of murine Mast cell protease-1 (mMCP-1) was measured in sera collected from female offspring, using a mMCP-1 Ready-SET-Go!® ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

A.4. Splenocyte re-stimulation assay

Re-stimulation of splenocytes for the analysis of *ex vivo* cytokine production was carried out as described earlier [36]. Splenocytes were isolated by smashing the spleen samples through 70-µm nylon cell strainer, and were counted and re-suspended in RMPI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin and 100 mg/mL streptomycin (culture medium) with or without 50 µg/mL OVA. After 5 days of culturing cells in 96-well U-bottom culture plates at 37°C in a humidified environment containing 5% CO2, supernatants were harvested and analyzed for determining the concentration of interleukin (IL)-4, IL-6, IL-10, IL-12p70, IL-13, IL-27, tumor necrosis factor (TNF)-α and interferon (IFN)-γ using ProcartaPlex multiplex protein assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

A.5. Isolation of RNA from Intestinal Samples for qRT-PCR

For mRNA isolation, proximal small intestine samples collected from mice were immediately frozen on dry ice and kept at −80°C until analysis. Tissue samples were weighed and homogenized in lysis buffer with 1:1 (w/v) ratio. Total RNA was isolated after DNAse treatment, using SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA), based on the manufacturer's instructions. Subsequently, the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to reverse-

transcribe the RNA into cDNA, using the T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).

Selected primers for zonula ocludence-1 (ZO-1), claudin-4 (CLDN-4), occludin (OCLD) and E-cadherin (Bio-Rad Laboratories, Hercules, CA, USA), together with iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) were used for qPCR (the primer efficiency for each tested primer was between 96 - 105%), and amplification was performed according to the manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA for each gene was normalized using the geometric mean of 2 reference genes, β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which are previously shown to be stable after DON exposure [37], and relative mRNA expression for each mouse was depicted as a fold change of the average of control group.

B. Vaccination model

The 4-weeks-old male offspring received vaccination one week after weaning, using Influvac (Abbott Biologicals B.V., Weesp, The Netherlands) from season 2015/2016, as previously described [38]. The mice received the primary and booster vaccinations by subcutaneous injections of 100 µL undiluted Influvac (containing hemagglutinin (HA) and neuraminidase antigens of three strains of influenza virus, in a dose equivalent to 30 µg/mL HA per strain, in total 90 µg/mL HA), or 10-times diluted vaccine (9 µg/mL HA). The booster vaccination was given 21 days after the primary vaccination. Sham group (negative control) which received injections of 100 µL PBS instead of vaccine was used to demonstrate the specificity of vaccine-induced response. Different doses of Influvac were tested in order to determine which dose would generate the immune responses with a larger effect size, so that the potential modulations by DON could be detected. Delayed-type hypersensitivity (DTH) was measured 9 days after booster vaccination, as described below. A day after DTH measurement, blood samples were collected by orbital extraction under inhalation anesthesia, followed by cervical dislocation. Tissue samples were collected for *ex vivo* analyses.

B.1. Antigen specific delayed-type hypersensitivity reactions

DTH reaction, as a model for cellular Th1-mediated immune reactivity, was determined 9 days after the booster vaccination, as described previously [38]. Undiluted Influvac (20 µL) was injected into the ear pinnae intradermally, under isoflurane-induced anesthesia. Ear thickness was measured in duplicate using digital-micrometer before injection and 24 hours thereafter, and the change in ear thickness was calculated by following formula:

ASR = (right ear (thickness at 1h - thickness at 0h)) - (left ear (thickness at 1h - thickness at 0h)

B.2. Re-stimulation of splenocytes with vaccine-loaded bone marrow-derived dendritic cells (BMDCs)

Bone marrow cells were isolated from femurs and tibias of healthy and untreated 5-week-old male mice born to control-fed mothers. Collected cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and Eagles minimum essential medium (MEM) non-essential amino acids (all from Gibco Life Technologies) in the presence of 10 ng/mL GM-CSF (Prosepec, The Netherlands) for 6 days to obtain immature BMDC (iDC) [38]. Induced iDCs were loaded with Influvac vaccine at a concentration of 0.9 µg/mL and incubated for 24 hours at 37°C, 5% CO2 to obtain matured DCs. DCs treated with medium were used as negative control. Spleen samples collected from vaccinated mice were smashed and splenocytes were isolated and resuspended in above mentioned supplemented culture medium, without GM-CSF. Freshly prepared splenocytes were cocultured with matured DCs at 10:1 ratio, in 96-well U-bottom culture plates for 5 days at 37°C, 5% CO2. Cell supernatants were collected and analyzed for the concentration of cytokines using ProcartaPlex multiplex protein assay kit (Invitrogen) according to manufacturer's instructions.

B.3. Serum concentration of specific antibodies

Blood samples collected from both male offspring were centrifuged (12000 RCF for 10 min) to collect the serum and were stored at −20°C until analysis. The serum samples were used to determine concentration of vaccine-specific immunoglobulin (Ig)G1 and IgG2a, according to the methods described previously [38, 39] by enzyme-linked immunosorbent assay (ELISA). Concentrations in test sera were calculated in arbitrary units (AU), relative to the standard curve of pooled plasma.

Flow cytometric analysis of spleen

Spleen samples collected from both male and female offspring were used for analyzing different immune cell populations by means of flow cytometry. Fresh splenocytes were isolated from spleens by methods described previously [36]. A complete list of labeled monoclonal antibodies used for staining the cells are shown in supplementary table 1. Cell viability was assessed by means of a fixable viability dye eFluor® 780 (eBioscience). For detecting intracellular transcription factors, Foxp3 Staining Buffer Set (eBioscience) were used for fixing and permeabilizing the cells, according to manufacturer's protocol. Results were collected with BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowLogic software (Inivai Technologies, Mentone, VIC, Australia).

Statistical Analysis

All data were analyzed by GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). For normally distributed data one-way ANOVA test was used, followed by a Bonferroni's multiple comparison post hoc test for selected comparisons, and for

not normally distributed and non-parametric data Kruskal-Wallis test was performed followed by Dunn's multiple comparisons test. Unpaired t test was used for comparing 2 means and in order to compare the ratio Fisher's exact test for differences between proportions was performed. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered statistically significant.

Sample size calculations

The required sample size was calculated separately for allergy and vaccination models, using G*Power v3.1.9. In allergy model, sample size was calculated based on ASR data available from previous experiments [24]. Control and OVA-treated groups were used to confirm the validity of the model, therefore based on data available from previous studies, a group size of n=4 was proven to be sufficient. The required number of animals in groups treated with OVA+CT was calculated as n=15, based on considering an outcome of 30% change in ASR as minimum relevant difference. The required sample size for vaccination model was calculated based on DTH data available from previous vaccination experiments [38]. Sham-treated groups were used for model validation, therefore a group size of n=3 was considered sufficient. The number of animals in vaccinated groups was calculated as n=9, based on considering an outcome of 10% change in ear thickness as minimum relevant difference. The power was set on 0.9 and α was corrected to the number of relevant comparisons. Based on the number of offspring required for both allergy and vaccination models, number of breeding female mice was calculated. Breeding success for female mice fed with control and DON-contaminated diets was calculated as 40% and 30%, respectively, based on results of a preliminary experiment in our group (unpublished data).

Results

Exposure to DON during pregnancy adversely affected the pregnancy outcome in mice. The breeding success in the control group receiving AIN93G diet was 39% (14 out of 36 mice) and in the female mice receiving DON-contaminated diet throughout the pregnancy period was 31% (22 out of 70 mice), though the difference was not statistically significant. The average litter size in DON-exposed mice was significantly smaller than control group (2.2 and 4.8, respectively, $p < 0.05$), but the ratio of male to female offspring was not significantly different between DON-exposed and control groups (0.7 and 1.1, respectively). The average weight gain from day 0 of pregnancy until the end of lactation period was 4.4 g in control-fed mice (n=14, SD=1.1) and 3.9 g in DON-fed mice (n=22, SD= 0.9), though the difference between groups was not statistically significant.

Maternal exposure to DON leads to enhanced allergic immune responses to OVA in the offspring.

Results of the acute allergic skin response (ASR), anaphylactic shock scores and serum concentration of OVA-specific immunoglobulins are shown in figure 2. In all animals sensitized with OVA+CT, ASR was significantly higher compared to PBS-treated mice (figure 2A), and OVA-specific IgG1, IgG2a, IgE and mMCP-1 levels in the serum was significantly increased (figure 2C, D, E and F), compared to non-(PBS) or OVA-sensitized mice. Maternal exposure to DON significantly enhanced ASR (figure 2A, $p<0.01$), and increased OVA-specific IgG1 and IgG2a (figure 2C and D, $p<0.05$) and IgE (figure 2E, $p<0.001$) levels in OVA+CT-sensitized animals, while no significant effect of DON on mMCP-1 concentrations was observed (figure 2F). Scoring the animals for development of shock symptoms 45 minutes after intradermal challenge revealed that 20% of OVA+CT-sensitized mice born to control-fed mothers showed mild-to-moderate shock signs, while in OVA+CT-sensitized mice born to DON-exposed mothers 60% of them had anaphylactic shock, with 20% showing severe symptoms (supplementary table 2). Thus, more animals developed anaphylactic shock symptoms, and severity of symptoms was enhanced in animals born to DON-exposed mothers, compared to those from control mothers (figure 2B, $p<0.05$). No significant difference in body temperature was observed between treatment and dietary groups (supplementary figure 1).

Interestingly, OVA-sensitized mice (without CT) born to DON-exposed mothers also developed shock symptoms and showed high ASR and vaccine-specific IgG2a production compared to non-sensitized groups and O VA-sensitized group born to control mothers (figures 2A and D), thought the differences in IgG2a levels are not significant due to small sample size ($p=0.09$). This indicates higher susceptibility of these animals to develop allergic response without receiving CT as adjuvant.

Maternal DON exposure induces limited effects on mRNA expression of junctional proteins in the duodenum of the offspring.

Direct exposure to DON is known to disrupt intestinal barrier integrity through changing the expression and localization of tight junction proteins [37]. Therefore, to gain more insight into possible intestinal integrity changes that might contribute to OVA allergic response, gene expression of junctional proteins in duodenum samples were measured in mice after oral OVA challenge. The expression of the target genes were normalized using 2 references genes, β-actin and GAPDH. There was no significant difference between different groups on mRNA expression of ZO-1, occludin (OCLD), E-cadherin and of claudin-4 (supplementary figure 2A, B, C and D).

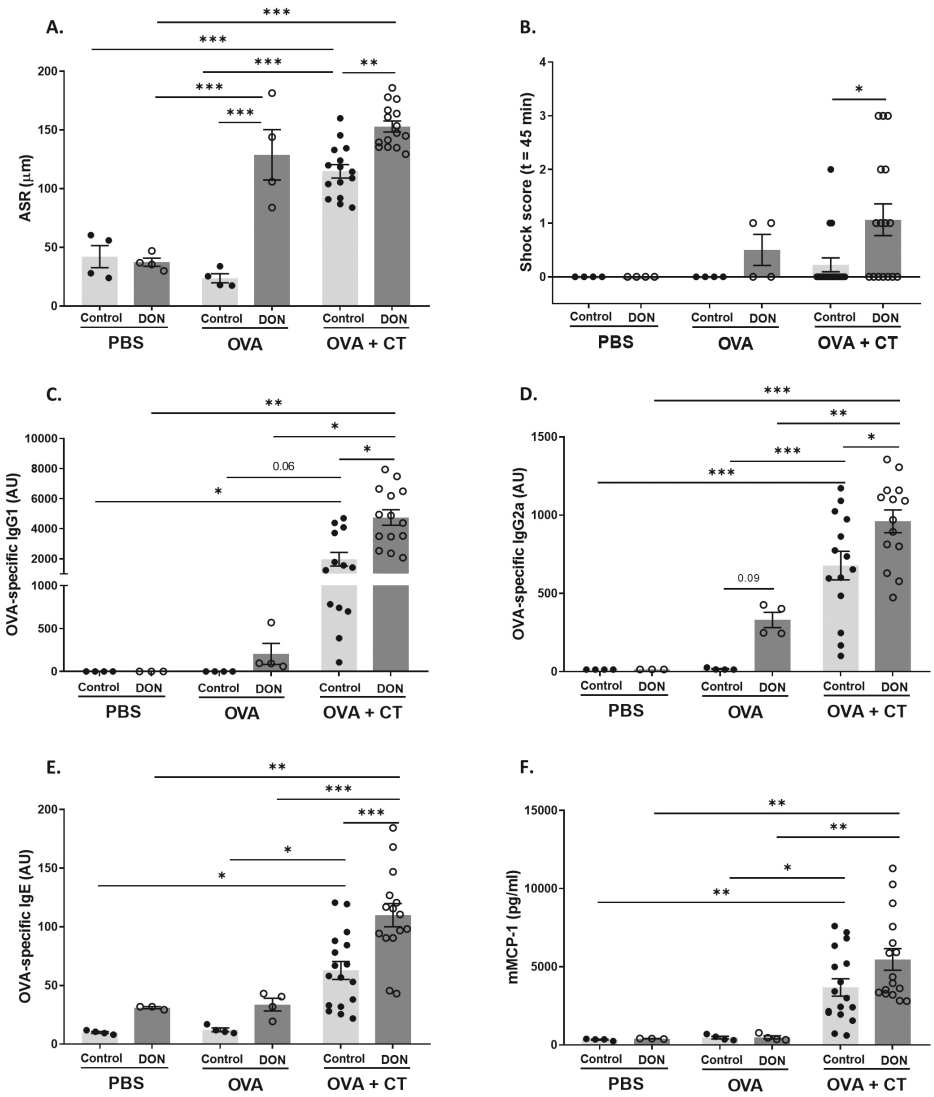

Figure 2. Maternal deoxynivalenol (DON) exposure enhanced ovalbumin (OVA)-specific allergic response and serum immunoglobulins (Ig) in the female offspring. Pregnant mice fed either a control or DON-contaminated diet (12.5 mg/kg) during pregnancy and lactation period. Female offspring received oral sensitizations with either phosphate-buffered saline (PBS), OVA, or OVA with cholera toxin (CT) after weaning. A week after the last sensitization, **A)** OVA-specific acute allergic skin response (ASR), measured as changes in ear thickness 1 hour after intradermal challenge; and **B)** accompanying anaphylactic shock score was determined 45 minutes after intradermal challenge with OVA. Serum levels of **C)** OVA-specific IgG1, **D)** IgG2a, **E)** IgE, and **F)** murine Mast cell protease-1 (mMCP-1) were measured 15 hours after oral gavage with ovalbumin. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistical differences.

Influvac-specific immune responses are attenuated in offspring born to DON-exposed mothers.

Results of Influvac-induced DTH reaction, as a model for *in vivo* cellular Th1 dependent immunity, showed a significant antigen-specific response to Influvac in vaccinated mice born to control mothers (figure 3A, $p<0.01$) and there was no significant difference between the 2 doses of influenza vaccine (9 and 90 µg/mL). However, the DTH response was significantly diminished in vaccinated mice born to DON-exposed mothers, compared to vaccinated mice from control mothers at both tested doses of vaccine ($p<0.05$).

As expected, all vaccinated mice had a significantly higher production of Influvac-specific IgG1 and IgG2a, compared to sham-treated groups (figure 3B and C). In vaccinated mice born to control mothers, administration of undiluted vaccine induced higher Influvac-specific IgG production compared to the diluted vaccine, while this effect was not observed in offspring of DON-exposed mice. When comparing groups receiving the higher dose of vaccine, mice born to DON-fed group had significantly lower serum levels of Influvac-specific IgG1 and IgG2a compared to those from the control-fed group ($p<0.05$ and $p<0.01$, respectively).

Maternal DON exposure reduces splenic Tbet+ T Helper 1 cells and regulatory T cells in the offspring.

The frequency of regulatory T cells (Treg) and T helper cells (Th1 and Th2) in isolated spleen samples were studied in both vaccination and allergy models, using flow cytometry (Figure 4A, gating strategy). In the OVA-induced food allergy model, after oral OVA challenge, OVA+CT sensitization in the offspring of non-exposed mice significantly increased the percentage of Treg cells, compared to PBS and OVA-sensitized groups (figure 4B, $p<0.01$), whereas no significant increase was detected in OVA+CT-sensitized group from DON-exposed mothers. Splenic Treg population in food allergic animals born to DON-treated mice was significantly lower than allergic mice from control mothers ($p<0.01$). No significant differences on percentages of $T1ST2^+$ Th2 cells and $CXCR3^+$ Th1 cells were observed between treatment and dietary groups (figure 4C and D). However, CT+OVA sensitization followed by OVA challenge causes a significant decrease in $Tbet^+$ Th1 cell population in mice born to control mothers (figure 4E, $p<0.05$ and $p<0.01$ compared to control and OVA groups, respectively). A similar level of reduction in $Tbet^+$ Th1 cell population was observed in all treatment groups from the offspring of DON-exposed mothers.

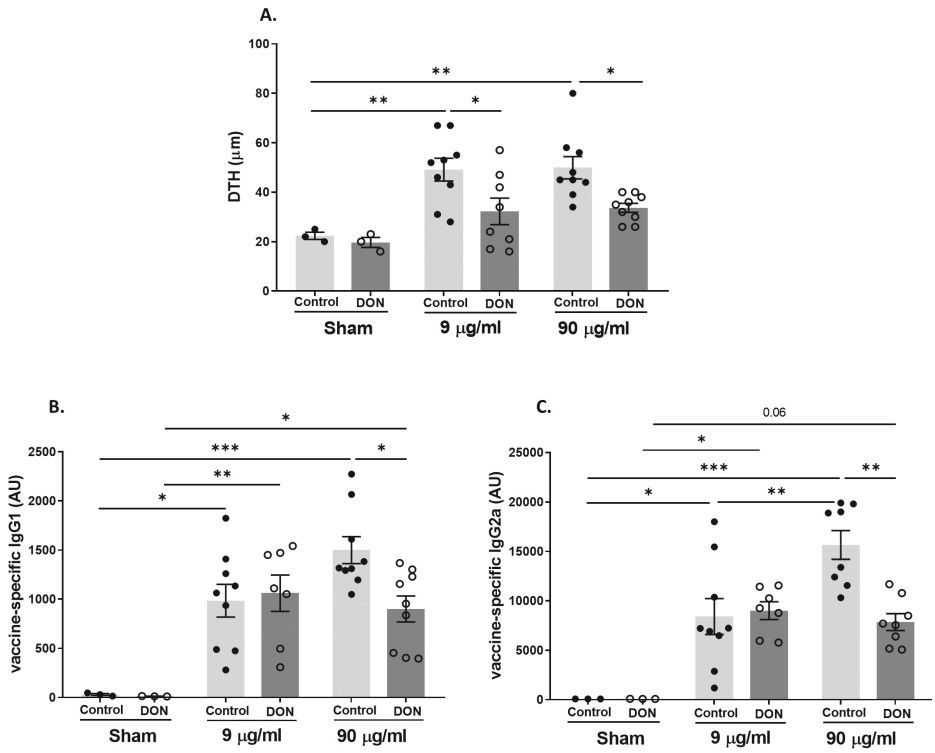

Figure 3. Maternal deoxynivalenol (DON) exposure reduced vaccine-specific immune responses in the male offspring. Pregnant mice fed either a control or DON-contaminated diet (12.5 mg/kg) during pregnancy and lactation period. Male offspring received subcutaneous injections of either phosphate-buffered saline (PBS, sham) or Influvac (9 and 90 µg/mL) after weaning. 10 days after the second injection, **A)** vaccine antigen-specific delayed-type hypersensitivity (DTH) was measured as changes in ear thickness 24 hours after intradermal challenge in Influvac; and **B)** vaccine antigen-specific immunoglobulin (Ig)G1 and **C)** IgG2a levels were measured in serum. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistical differences.

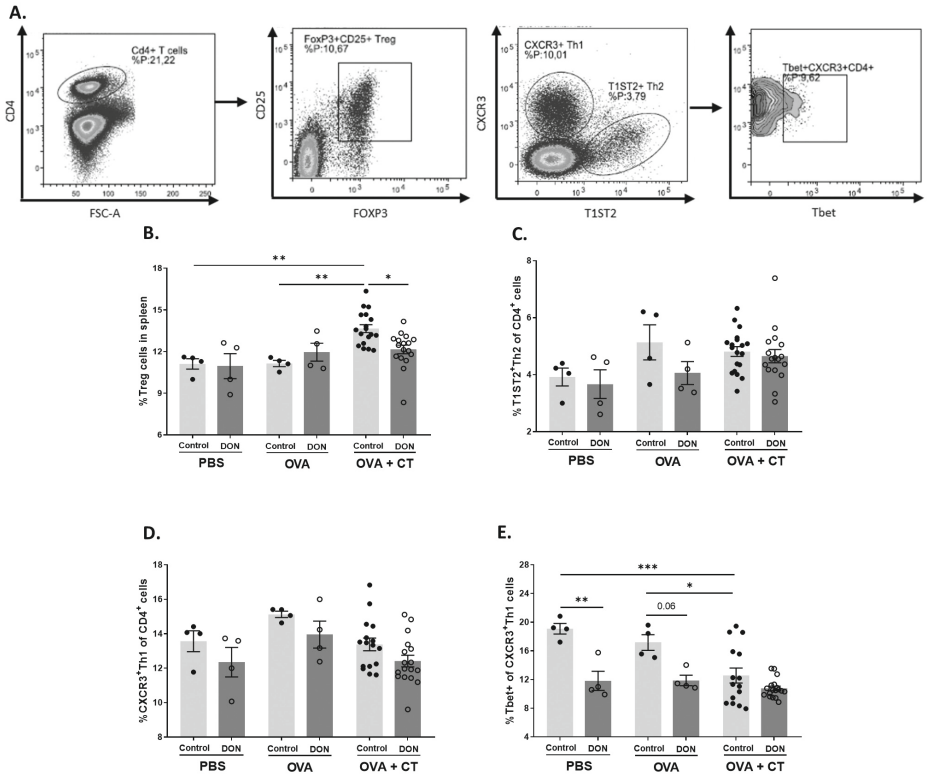

Figure 4. Maternal deoxynivalenol (DON) exposure modulated the splenic T cell populations in the female offspring. Pregnant mice fed either a control or DON-contaminated diet (12.5 mg/kg) during pregnancy and lactation period. Female offspring received oral sensitizations with either phosphate-buffered saline (PBS), ovalbumin (OVA), or OVA with cholera toxin (CT) after weaning. A week after the last sensitization, female offspring were sacrificed and spleen samples were used for flow cytometric analysis of T cell subpopulations. **A)** Gating strategy for selecting different subtypes of T cell out of live $CD4^+$ cells in spleen. Percentages of **B)** $CD25^+Foxp3^+$ regulatory (Treg) cells**; C)** $T1ST2^+$ Th2 cells; **D)** $CXCR3^+$ Th1 cells; and **E)** $Tbet^+$ cells out of $CXCR^+$ Th1 cells; Data are presented as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate statistical differences. (FOXP3 (fork-head box P3), CXCR3 (C-X-C Motif Chemokine Receptor 3), T-bet (T-box expressed in T cells, TBX21)).

Similar results were observed in the splenic T cell populations of vaccinated animals (figure 5). Vaccination with different concentrations of Influvac had no significant effect on Treg cells, however significant reduction of splenic Treg cells was observed in all treatment groups of the offspring of DON-treated mice (figure 5A). Although no significant differences for Th2 and Th1 cell populations were observed between treatment and dietary groups (figure 5B and C), vaccination with higher concentration of Influvac significantly increased $Tbet^+$ Th1 cells in the spleen of the offspring born

to control mice (figure 5D). However, in animals born to DON-exposed mothers no significant increase in Tbet$^+$ Th1 cell after vaccination was detected.

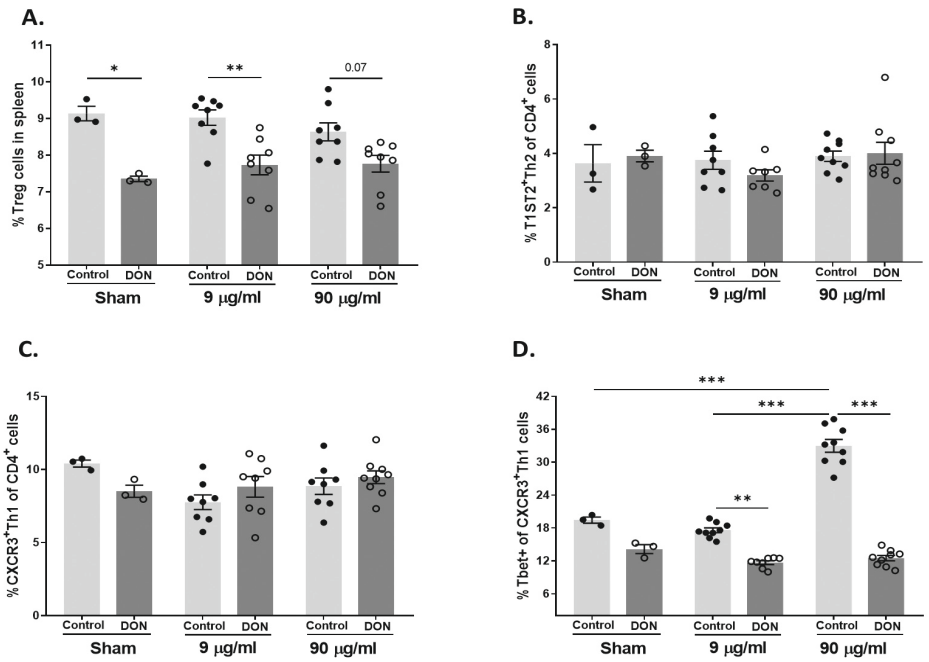

Figure 5. Maternal deoxynivalenol (DON) exposure modulated the splenic T cell populations in the male offspring. Pregnant mice fed either a control or DON-contaminated diet (12.5 mg/kg) during pregnancy and lactation period. Male offspring received subcutaneous injections of either phosphate-buffered saline (PBS, sham) or Influvac (9 and 90 μg/mL) after weaning. 10 days after the second injection, male offspring were sacrificed and spleen samples were used for flow cytometric analysis of T cell subpopulations. Percentages of **A)** CD25$^+$Foxp3$^+$ regulatory (Treg) cells**, B)** T1ST2$^+$ Th2 cells; **C)** CXCR3$^+$ Th1 cells, and **D)** Tbet$^+$ cells out of CXCR$^+$ Th1 cells, out of live CD4$^+$ cells in spleen; Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistical differences. (FOXP3 (fork-head box P3), CXCR3 (C-X-C Motif Chemokine Receptor 3), T-bet (T-box expressed in T cells, TBX21)).

Maternal DON-exposure decreases ex vivo Th1-related cytokine production.

The antigen-specific cytokine expression from splenocytes isolated from the offspring were evaluated by *ex vivo* re-stimulation of the cells with either OVA in allergy model, or Influvac-treated DCs in vaccination model. OVA-specific IL-6 and IL-27 release were not affected by maternal DON-exposure or sensitization status of the offspring (figures 6C and 6F). Although sensitization with OVA+CT followed by OVA challenge induced significant elevation in IL-12p70, IL-13, IL-4 and IL-10 release, the release of these cytokines was not further affected by maternal exposure to DON (figures 6B, D, E and G). In the offspring of control mothers, IFN-γ release in splenocyte supernatant

was significantly lower in the offspring receiving OVA+CT, compared to OVA-sensitized group ($p<0.001$), whereas in the offspring of DON-exposed mothers the concentration of IFN-γ was as low as OVA+CT sensitized mice (figure 6A). Maternal exposure to DON also significantly increased TNF-α release from cells of OVA+CT sensitized mice born to DON-exposed mothers, compared to the cells of animals from control-fed mothers (figure 6H).

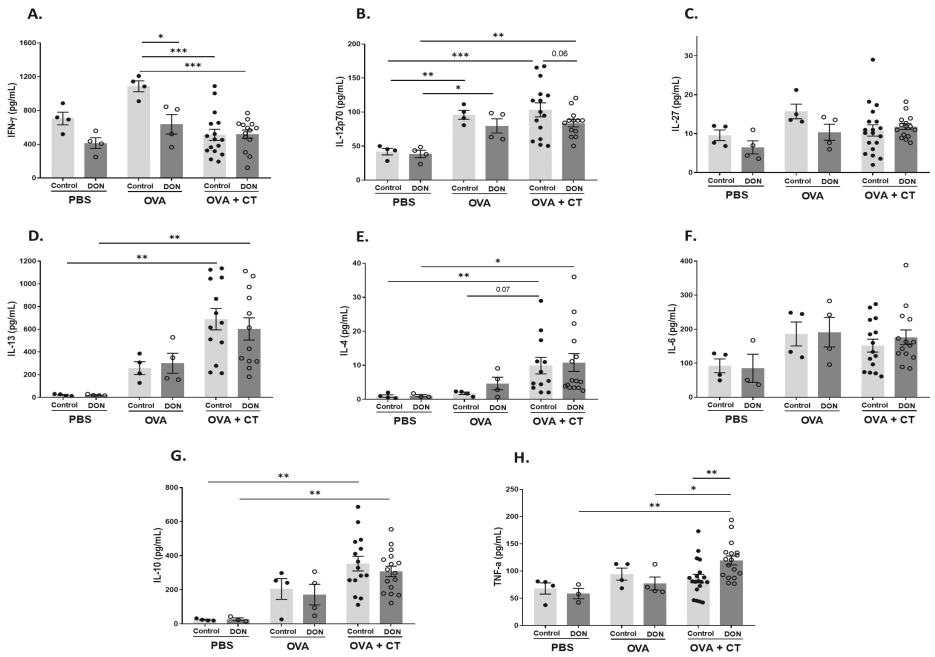

Figure 6. Maternal deoxynivalenol (DON) exposure modulated the cytokine production of ovalbumin (OVA)-stimulated splenocytes of female offspring *ex vivo*. Pregnant mice fed either a control or DON-contaminated diet (12.5 mg/kg) during pregnancy and lactation period. Female offspring received oral sensitizations with either phosphate-buffered saline (PBS), OVA, or OVA with cholera toxin (CT) after weaning. A week after the last sensitization, female offspring were sacrificed and collected splenocytes were re-stimulated *ex vivo* with OVA. **(A-H)** Interféron (IFN)-γ, interleukine (IL)-12p70, IL-27, IL-13, IL-4, IL-6, IL-10 and tumor necrosis factor (TNF)-α concentrations. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistical differences.

In male offspring born to control-fed mice, production of all tested cytokines from isolated splenocytes were significantly increased in vaccinated groups (figure 7). The effect of the higher dose of Influvac was more prominent in release of IFN-γ and IL-27, while the effect of the lower dose was more prominent on IL-13, Il-6 and IL-10. Maternal DON exposure had no significant effect on release of IL-4, Il-13, IL-6, IL-10 and TNF-α by splenocytes from vaccinated mice, compared to the mice born to control

mothers, however when comparing groups vaccinated with the higher dose of Influvac, splenocytes of mice from DON-fed mothers had a significantly lower production of Th1-related IFN-γ, IL-12p70 and IL-27 (figure 7A, B and C).

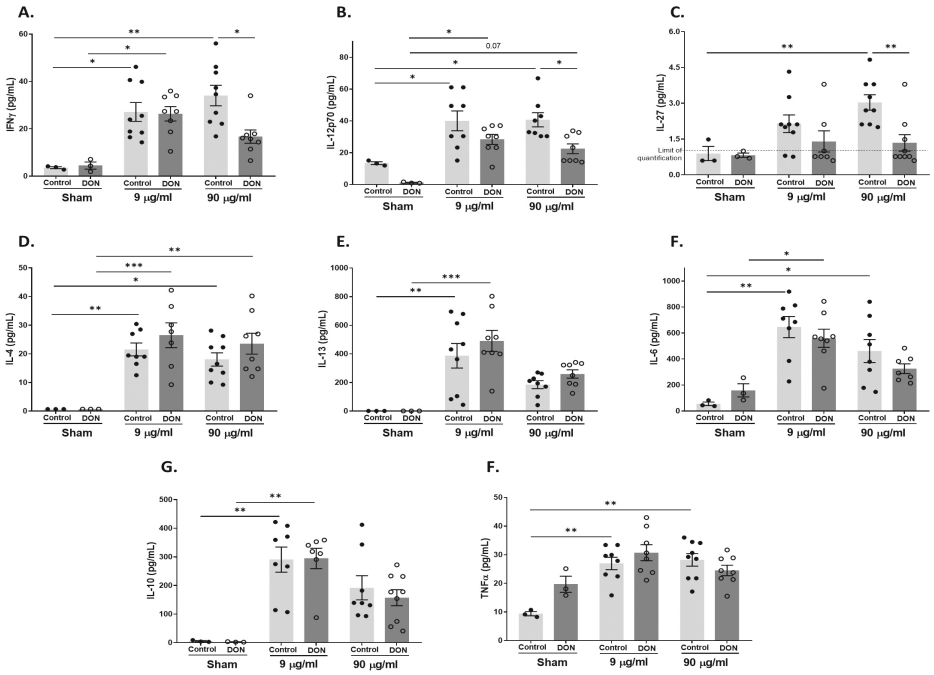

Figure 7. Maternal deoxynivalenol (DON) exposure modulated the cytokine production of splenocytes of male offspring co-cultured with influenza-loaded bone marrow-derived murine dendritic cells (DCs) *ex vivo*. Pregnant mice fed either a control or DON-contaminated diet (12.5 mg/kg) during pregnancy and lactation period. Male offspring received subcutaneous injections of either phosphate-buffered saline (PBS, sham) or Influvac (9 and 90 µg/mL) after weaning. 10 days after the second injection, male offspring were sacrificed and collected splenocytes were co-cultured with influenza-loaded bone marrow-derived DCs from healthy, untreated mice. **(A-H)** Interferon (IFN)-γ, interleukine (IL)-12p70, IL-27, IL-13, IL-4, IL-6, IL-10 and tumor necrosis factor (TNF)-α concentrations. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate statistical differences.

Discussion and conclusion

There are increasing evidence supporting the significant role of immunotoxicity in development of non-communicable diseases [6]. Therefore, studying earl-life toxicity induced by regularly occurring immunotoxins, such as mycotoxins, is of greatest importance. DON, a highly prevalent food contaminant, is a potent immunotoxic agent but information on its effects on immune development in early-life is scarce. Administration of 4.42 mg DON/kg diet to pregnant pigs during second half of pregnancy was shown to induce no significant histomorphological changes in the lymphoid organs of the fetuses [40]. However, intravenous administration of DON (300 µg in 500 mL of infusion) in pregnant pigs at the end of gestation altered the prevalence of different lymphocyte subtypes in the blood of piglets after birth [27], indicating a disturbed and unbalanced immune system in these animals. The present study is the first to investigate the consequences of developmental immunotoxicity of DON in later stages of life.

The allergic immune response to OVA was studied in female offspring born to either control or DON exposed mothers. When investigating OVA+CT-sensitized animals, significant increases in ASR and average anaphylactic shock scores upon intradermal challenge were observed in the offspring born to DON-exposed mothers as compared to control mothers, indicating that these animals had more severe allergic reactions. OVA-specific serum IgGs and IgE levels were more elevated in allergic mice born to DON-fed animals, which further confirms the higher intensity of OVA-specific allergic immune responses due to maternal exposure to DON. Murine MCP-1 is another serum marker for mast cell-dependent intestinal inflammation in murine allergy models, and can alter the permeability of the small intestine by breaking intercellular tight junction connections [41]. Production of mMCP-1 was increased in OVA+CT-sensitized mice, however maternal DON-exposure had no significant effect on soluble mMCP-1 levels. Interestingly, when comparing OVA-treated animals (without CT), an increase in ASR and serum IgGs production after OVA challenge was detected in mice born to DON-exposed mothers, though the level of OVA-specific IgE levels were not affected. This observation indicates that these animals might be more prone to develop allergy. Treatment with the mucosal adjuvant CT disrupts the intestinal epithelial cell layer and facilitates allergy development to OVA [24], while OVA treatment without CT is not expected to induce significant allergic reactions. Therefore, these observations indicate that early-life DON exposure can facilitate allergy development in the absence of CT. Larger sample size for OVA-treated group is required for definite conclusion, however our results indicate that early-life exposure to DON worsens the OVA-sensitization response in mice.

Results of an earlier study on adult mice showed that DON can mimic the effect of CT in allergic sensitization [24]. Oral administration of DON together with whey protein facilitated food allergy induction, indicated by increased ASR values and serum levels of whey-specific IgE, and the production of IL-5 and IL-13 in re-stimulated splenocytes [24].

A possible mechanism responsible for increased susceptibility to develop allergy after direct exposure to DON could be the induction of epithelial cell stress and intestinal barrier disruption, through changing the expression of junctional proteins, leading to increased intestinal permeability [24, 37]. In the current study comparing the mRNA expression of junctional proteins in the duodenum of mice born to DON-exposed mothers to those born to control mothers revealed no significant difference. These observations might be in part due to the indirect exposure of the offspring to DON. Furthermore, the offspring received control diet from a week before weaning till the end of the experiment; therefore, any modifications in the intestine induced by early exposure to DON could possibly be restored during the DON-free period. Nevertheless, further analyses of protein expression of tight junctions can shed more light on potential DON-induced alterations in the intestine of the offspring.

In a mouse model of house dust mite -induced allergic asthma, acute and subacute oral treatment with DON significantly enhanced the inflammatory responses, by increasing the infiltration of $CD3^+CD4^+$ helper T cells and IgE^+ B cells and enhancing production of IL-4, IL-5 and IL-13 in local lymph nodes [42]. In the current study, there was no significant difference in the population of splenic Th2 cells between different groups, and the production of Th2-mediated cytokines, such as IL-13 and IL-4, from re-stimulated splenocytes of OVA-sensitized animals born to control and DON-exposed mothers was not significantly different. However, production of TNF-α, a pleiotropic cytokine, was increased in OVA-sensitized animals born to DON-exposed mothers, compared to OVA-sensitized mice from control mothers. TNF-α is shown to be required for antigen-specific IgE and Th2-type cytokines production in allergic rhinitis in mice [43], and is necessary for regulating production of IL-4 and IL-13 [44]. Furthermore, an overall significant reduction in $Tbet^+$ Th1 cells and downregulation of Th1-mediated IFN-γ production was observed in the offspring of DON-exposed mothers in the OVA-specific allergy model. Previous studies have reported that upregulation of Tbet expression directly correlates with inhibition of IL-4-induced IgE and IgG class switching in B cells [45]. A reduction of Tbet expression is associated with the development of allergy in mice [34], as a reduction in Th1-mediated immune capacity can disturb the balance between Th1- and Th2-mediated immunity and shift the balance toward Th2-mediated responses, subsequently rendering the immune system more susceptible to allergy development. In addition, it has been shown that higher production of IFN-γ in mice receiving OVA leads to the induction of tolerance, while depletion of IFN-γ results in failure to control Th2 cell responses to allergens [46, 47]. Thus, although the percentage of Th2 cells and concentration of Th2-mediated cytokines are not significantly affected by DON, it could be suggested that the increased intensity of allergic reactions and production of OVA-specific immunoglobulins is due to a lack of a proper Th1-mediated immune response which would otherwise neutralize the Th2-mediated allergic responses. However, further investigation with a focus on earlier stages of allergy development can provide a better insight to the mechanisms behind these observations. Similarly, a reduction in

Tbet$^+$ Th1 cells and Th1-mediated cytokines was observed in OVA-treated mice (without CT) born to DON-exposed mothers, which may, in part, explain the increased ASR and vaccine-specific IgG production in these animals. T cell-mediated mechanisms may play a more prominent role in induction of observed allergic reactions in these animals [48, 49], as the production of IgE in these animals was not significantly affected by maternal DON exposure. However, further investigation with larger sample size is required to confirm these findings and identify potential non-IgE-mediated mechanisms involved in induction of the observed hypersensitivity reactions.

A suppressed immune response to influenza virus antigens was observed in vaccinated mice born to DON-exposed mothers, indicated by a reduced DTH response and serum levels of vaccine-specific IgGs. Similar to the observations in the allergy model, a significant reduction in the percentage of Tbet$^+$ Th1 cells in the spleen, and the production of Th1-mediated cytokines, such as IFN-γ, IL-12p70 and IL-27 were observed in these animals. IFN-γ plays a key role in anti-microbial immunity and a decrease in the capacity of IFN-γ production can reduce host resistance to pathogens and increase susceptibility to specific bacterial and viral infections [50, 51]. Therefore, lower Th1-mediated responses can, in part, explain diminished vaccine antigen-specific immune responses in the offspring of mice fed with DON-contaminated diet. There is an altered immune response to enteric and respiratory reovirus infection upon DON administration in adult mice [25, 26]. Reovirus-specific IgA levels in serum of mice receiving one-time oral gavage of 10-25 mg/kg of body weight DON were elevated and IFN-γ production in response to reovirus was suppressed, which resulted in transiently increased severity of the viral infection [25, 26]. Moreover, feeding pigs with DON-contaminated diets (3.5 mg/kg of diet) impaired the immune response after vaccination with PRRSV (porcine reproductive and respiratory syndrome virus) live attenuated vaccine, by decreasing antibody response to vaccination and negatively affecting the replication of vaccinal virus [52]. Although the dose of DON, route of administration and exposure duration vary in above-mentioned studies, these observations correspond with the suppressed Th1-mediated immune capacity and Th2-skewing properties of DON.

There was a downregulation of CD25$^+$Foxp3$^+$ regulatory T cells in the spleen in both allergic and vaccinated mice born to DON-exposed mothers, compared to the offspring of control-fed mothers. A similar decrease in regulatory T cells in the blood leukocytes of piglets was reported after intravenous administration of DON during pregnancy in pigs [27]. Regulatory T cells have a prominent role in vaccination responsiveness [53, 54] and allergy development [55]. Treg populations actively prevent hypersensitive immune responses [56]. CD4$^+$CD2S$^+$Foxp3$^+$ Treg cells can suppress Th2 responses to inhaled and food allergens [57], and it is suggested that the function of Treg cells may be impaired in allergic patients [58]. Therefore, a reduced Treg cell population might contribute to the imbalanced immune responses observed in the present study.

This study was designed based on previous experiments with the murine models of pregnancy and OVA-specific food allergy using female C3H/HeOuj mice [59]. The OVA-specific food allergy model used in this study was previously optimized using female mice to obtain the desired allergic responses [34, 59, 60], while for vaccination responsiveness murine models are developed using both male and female mice [61, 62]. Therefore, female offspring were used for OVA-specific food allergy model and male offspring were used for vaccination model, to obtain the desired immune responses. However, regardless of the immune challenging model, similar Th2-skewing effects were observed in both male and female offspring, thus it can be concluded that the effect of maternal exposure to DON on disturbing the immune balance is consistent in both sexes. Nevertheless, it is indeed highly relevant to further investigate both models in both sexes to compare the sensitivity of male and female offspring to DON-induced effects.

Although there are regulations set to limit the level of DON concentration in human food products (1-2 mg/kg of food) [29], masked and modified forms of DON, which are not detected by established standard methods, may lead to underreporting of mycotoxin levels in food products [63]. Moreover, humans are likely exposed to varying levels of the mixtures of mycotoxins present in the different food products on a daily basis, which can have additive to synergistic toxic effects [64, 65], and make it difficult to estimate the exact exposure levels. Thus, investigating the immunomodulatory effect of DON at a wide range of concentrations, including the concentrations above the established limits can be considered as highly relevant. This study focused on the early-life exposure to DON through maternal diet during the entire period of pregnancy and lactation to mimic the real-life human situation, as the exposure to DON through diet is expected to remain similar throughout this period. Nevertheless, studying the effect of DON exposure at specific developmental stages in early-life would provide a comprehensive understanding of its mode of action and would help to come up with more effective strategies to prevent DON-induced toxicities.

In conclusion, results of the current study show that early-life dietary exposure to the food contaminant DON can adversely influence immune maturation and development during early-life in the offspring. As a consequence, the immune system in the offspring is skewed toward an imbalanced state, resulting in an increased allergic immune response to food allergens and a decreased immune response to influenza virus vaccine. The observed effects can be, in part, due to the suppressed Th1-mediated immune capacity and Th1/Th2 imbalance toward Th2 in these animals. However, further investigation is required to fully understand the exact mechanisms involved in DON-induced developmental immunotoxicity. Considering the difficulty of eliminating mycotoxin exposure in humans, as well as the impact of climate change on increasing the contamination of agricultural products with Fusarium mycotoxins [66], it is important to be aware of the potential harmful effects of DON present in food

products and understand the exact mechanisms of its toxicity, in order to efficiently prevent or attenuate its adverse effects.

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Author contributions
The author's responsibilities were as follow: BL, SB, AH, NST: designed the research; BL, SB, AH, JG, ADK, GF: supervised data interpretation; NST, ST: conducted the *in vivo* experiment; NST: analyzed data and wrote the paper; All authors provided critical intellectual input for data interpretation, read and approved the final manuscript.

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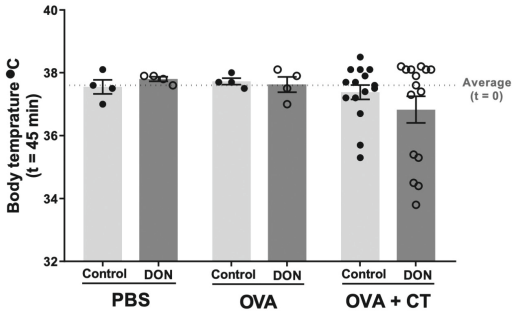
Supplementary content

Supplementary table 1 List of antibodies used in flowcytometry analysis.

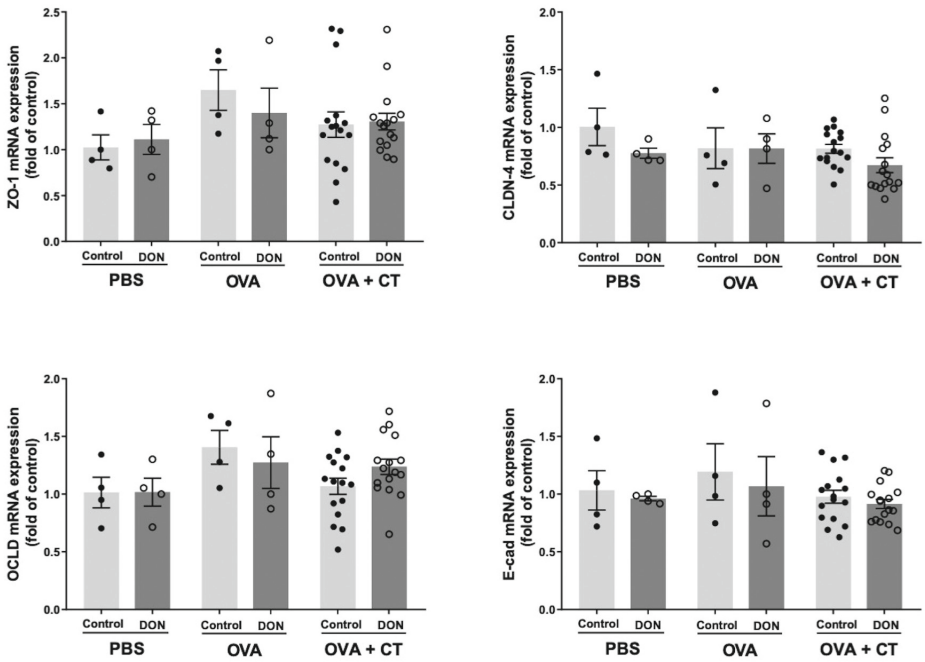
Marker	Label	Catalog number	Antibody ID	Dilution
CD4	BV510	100553	AB_2561388	1:160
CD69	PE-Cy7	25-0691	AB_469637	1:640
T1/ST2	FITC	101001F	AB_947549	1:200
CXCR3	PE	12-1831	AB_1210734	1:100
Tbet	Alexa Fluor647	644803	AB_1595573	1:1600
Gata3	PerCP-eFluor710	46-9966	AB_10804487	1:1000
CD25	PerCP-Cy5.5	45-0251	AB_914324	1:1200
CD196 (CCR6)	PE	129804	AB_1279137	1:640
FoxP3	FITC	11-5773	AB_465243	1:100
RorgT	Alexa Fluor647	562682	AB_2687546	1:400

Supplementary Table 2. Number and percentage of animals developing shock symptoms 45 minutes after intradermal skin challenge with ovalbumin.

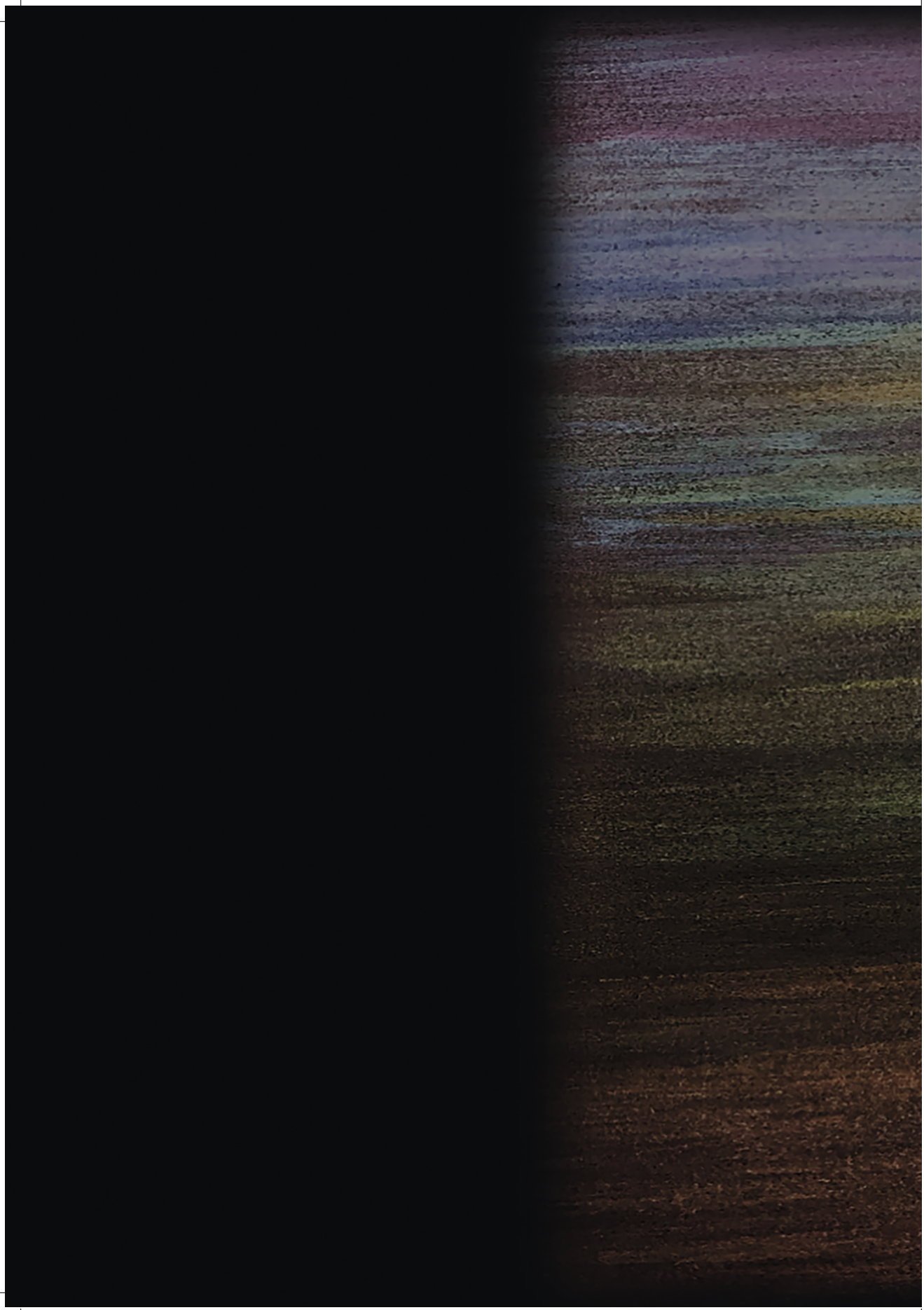
Maternal diet	**Control**			**DON**		
Sensitization	**PBS**	**OVA**	**OVA+CT**	**PBS**	**OVA**	**OVA+CT**
Mice with shock score ≥ **1 (%)**	0/4 (0%)	0/4 (0%)	3/15 (20%)	0/4 (0%)	2/4 (50%)	9/15 (60%)
Mice with shock score ≥ **2 (%)**	0/4 (0%)	0/4 (0%)	1/15 (6.6%)	0/4 (0%)	0/4 (0%)	5/15 (33.3%)
Mice with shock score ≥ **3 (%)**	0/4 (0%)	0/4 (0%)	0/15 (0%)	0/4 (0%)	0/4 (0%)	3/15 (20%)



Supplementary figure 1. Body temperature (°C) of female offspring. Pregnant mice fed either a control or deoxynivalenol (DON)-contaminated diet (12.5 mg/kg) during pregnancy and lactation period. Female offspring received oral sensitizations with either phosphate-buffered saline (PBS), OVA, or OVA with cholera toxin (CT) after weaning. A week after the last sensitization, drop in body temperature was determined 45 minutes after intradermal challenge with OVA in the ear. The average body temperature at time 0 before intradermal challenge was 37.7 °C (±0.6 °C), which is determine the graph with dotted line. Data are presented as mean±SEM.



Supplementary figure 2. Effect of maternal DON exposure on mRNA expression of intestinal barrier integrity molecules in the offspring. Relative mRNA expression (fold of control, normalized to β-actin) of **A)** zonula occludens-1 (ZO-1), **B)** claudin-4 (CLDN-4), **C)** occludin (OCLD), and **D)** E-cadherin (E-cad). Data are presented as mean ± SEM.



Chapter 7

General Discussion and Conclusion

General discussion

Fusarium fungal species are the most prevalent contaminants of cereal grains worldwide [1], producing several toxic metabolites known as mycotoxins. The Fusarium mycotoxins deoxynivalenol (DON), T-2 toxin and zearalenone (ZEN) are recognized as the major mycotoxins occurring in food products [2]. Ingestion of grain-based products contaminated with these mycotoxins can adversely affect the function of the gastrointestinal tract, reproductive organs and the immune system, and depending on the concentration and duration of exposure, it can lead to acute toxicities with even high morbidity or chronic diseases [3].

A major problem associated with mycotoxin exposure is the long-term ingestion of low non-toxic levels of these toxins which may cause metabolic, physiologic, and immunologic disturbances at the end [3]. Additionally, chronic exposure to DON and other Fusarium mycotoxins alters host-pathogen interactions and exacerbates infections with parasites, bacteria and viruses across a wide range of species, including humans [4]. Exposure to mycotoxin-contaminated food during early stages of life is associated with various adverse health outcomes, such as skeletal malformation, neural tube defects, growth retardation and neonatal jaundice in mice and rats [5, 6]. However, the long-term effects of mycotoxin exposure in early life are not fully understood yet. In this present thesis, we investigated the impact of Fusarium mycotoxins, with a special focus on DON, on immune functioning, pregnancy outcome, placental structure and function, as well as the potential long-term effects on the immune system in the offspring.

Part 1: DON-induced Immune effects

The immune system is extremely sensitive to DON and its ingestion can alter immune responses even at very low nontoxic levels making it an immunotoxic agent [7, 8]. Depending on the concentration and duration of exposure either immuno-suppressive or immunostimulatory effects can be induced by DON [9]. High doses of DON cause immunosuppressive effects due to apoptosis of leukocytes, while immunostimulatory effects are seen after exposure to low doses [10-12]. Several molecular mechanisms have been suggested to play a role in DON-induced immunotoxicity, such as protein synthesis inhibition and activating mitogen-activated protein kinases (MAPKs), through a mechanism known as the ribotoxic stress response [13]. Furthermore, DON can induce endoplasmic reticulum (ER) stress, and even mitochondrial dysfunction in immune cells [14].

In **chapter 2** of the present thesis, the immunomodulatory effects of chronic exposure to DON on Th1 responses was studied in a murine vaccination model, which is a validated *in vivo* model for investigating cellular and humoral immune responses with a focus on Th1-mediated immunity [15, 16]. Addition of DON to the diet of vaccinated mice

led to a significant drop in the frequency of splenic B cells and humoral immunity, as a reduction in antigen-specific serum immunoglobulin levels was detected. In addition, DON exposure decreased splenic Tbet$^+$ Th1 cells and induced a significant reduction in IFN-γ secretion by splenocytes after *ex vivo* re-stimulation. These results are in line with previous studies, which demonstrated that DON exposure transiently diminished the host's response to reovirus by suppressing IFN-γ and increasing IL-4 mRNA expression in Peyer's patches, and consequently suppressing type-1 IFN-mediated responses [17, 18]. Lower Th1-mediated responses can in part explain the diminished vaccine antigen-specific immune responses in the mice fed with a DON-contaminated diet.

Low concentrations of DON alter cytokine secretion capacities of neutrophils [19], and reduce T-cell proliferation and activation in porcine polymorphonuclear cells [20], as well as in human mononuclear cells [21, 22]. Although several studies have demonstrated toxic effects of DON on different immune cells, the effect of DON on the interactions between different immune cells is not fully understood. Considering that dendritic cells (DC) play a key role in generating adaptive immune responses [23], the effects of DON on the capacity of DC to activate T cells and subsequent effects on B-cell activation, using an *in vitro* allogeneic coculture model was investigated (**chapter 3**). DON exposure of LPS-stimulated human monocyte-derived DC inhibited the up-regulation of DC maturation and activation markers HLA-DR and CD80, and significantly prevented LPS-induced production of IL-10 (1-4 μM DON) and IL-12p70 (2-4 μM DON), confirming the earlier observations in human and porcine DC studies [10, 24, 25]. Remarkably its effect on LPS-induced IL-6 production was less prominent.

DCs are crucial for T-cell priming in lymph nodes [23]. Depending on the signals provided by DCs, T cells differentiate into a specific subset, leading to active immune responsiveness or immunological tolerance [26]. The profile of cytokine secretion by activated DCs is a crucial factor in polarization of T cells; IL-12 favors Th1 differentiation by priming naïve CD4$^+$ T cells for high IFN-γ production [27], and other Th1-drived cytokines from Tbet$^+$ T cells [28], while IL-6 contributes to Th2-polarization and inhibits Th1 differentiation by initiating the production of IL-4 [29, 30]. IL-10 is a well-known anti-inflammatory and regulatory mediator and negatively regulates IL-12 levels by inhibiting the upregulation of costimulatory molecules and IL-12 production [31]. Thus, modification in DC activation status and cytokine profile is consequently reflected in T-cell activation and polarization. In the DC-T cell coculture model described in **chapter 3**, DON exposure of LPS-stimulated DC diminished the capacity of these cells to activate T cells and Th1-polarization, hence skewing T cell responses toward Th2, which might promote allergy development. Phenotype analysis of T cells after coculture revealed a significant increase in percentages of GATA3$^+$ Th2 cells due to LPS+DON exposure in DC. Similarly, mRNA expression of GATA3 was significantly increased, while mRNA expression of Tbet was decreased in T cells cocultured with LPS+DON-treated DC. Moreover, production of the Th2-associated cytokine IL-13 was significantly increased

in T cells cultured with LPS+DON-exposed DC. These results indicate that DON-exposed DCs are Th2-biased and favor Th2 differentiation. Interestingly, exposing LPS-stimulated DC to the lowest concentration of DON (1 μM) increased IFN-γ production, while the higher concentrations of DON significantly down-regulated IFN-γ production. The increase in IL-13 production after DON exposure, and the dual effect of DON on IFN-γ, may be partially explained by the alterations in the ratio of cytokines produced by DC, IL-10, IL-12 and IL-6, at different DON concentrations. These observations are in line with results of our vaccination study in mice (**chapter 2**) and further confirms the Th2-skewing effects induced by DON. In addition, it was demonstrated that the production of antigen-specific Th1-associated cytokines IFN-γ and IL-27 from splenocytes of mice vaccinated with Influvac (receiving control diet) was significantly reduced after *ex vivo* restimulation by Influvac + DON-treated murine bone marrow-derived DC, compared to Influvac-treated DC, while concentrations of IL-13 were not changed (**chapter 3**). Although DON exposure significantly decreased the production of IL-10 in splenocytes of the vaccinated mice (**chapter 2**), no significant effect of DON on the percentage of $FoxP3^+$ regulatory T cells was observed in either the murine vaccination study or the human *ex vivo* DC-T cell coculture models, indicating that mechanisms other than changes in regulatory T-cell population may play a role in the DON-induced imbalanced Th1/Th2 responses as well (**chapter 2 and 3**).

To understand the mechanism of DON-induced immunotoxicity in DC, the mRNA expression of several key markers involved in the DC activation pathway were analyzed. Toll-like receptor (TLR)4 is a critical receptor and signal transducer for LPS, and TLR4-dependent pathways are essential for DC activation and maturation upon LPS stimulation [32]. Cytoplasmic adapter protein MyD88 is essential for TLR4-associated cytokine production [32]. CD11b (ITGAM) is another important protein in TLR4-dependant signaling pathways that positively regulates LPS-induced DC activation and CD11b deficiency leads to impaired DC-mediated T cell activation [33]. DON exposure significantly altered the mRNA expression of TLR4, MyD8 and CD11b in LPS-stimulated DC (**chapter 3**). Previous studies have reported that in $MyD88^{-/-}$ DC, LPS-induced IL-12 production is abolished [32], and DCs support Th2-polarization and elevated IL-4 production [34]. Thus, DON-induced modulations in TLR4/MyD88-associated signaling pathways may, partly be responsible for altered T cell-polarizing capacity of DC and diminished Th1-associated immune responses.

DC signalling is a prerequisite for T cell-dependent activation of naïve B cells and initiation and regulation of immunoglobulin synthesis [35, 36]. As described in **chapter 3**, coculturing naïve B cells and T cells with LPS-stimulated DC induced differentiation of naïve $CD27^-CD38^-$ B cells to $CD27^+CD38^+$ plasma cell and production of IgM and IgG. This B cell activation and differentiation depended on both DC and T cells, as coculturing B cells with DC or T cells alone did not induce any differentiation. Stimulated DC and DC-activated $CD4^+$ T cells interact with B cells and induce IgM, and in later stages, IgG

production [37, 38]. DON exposure of LPS-stimulated DC significantly inhibited B cell differentiation to plasma cells and IgM production (**chapter 3**). These observations are in line with previous studies reporting a significant reduction in immunoglobulin production by stimulated peripheral blood lymphocytes after being directly exposed to DON [39, 40], even though in the coculture system used in **chapter 3,** B cells and T cells are not directly exposed to DON. The differentiation of naïve B cells in a coculture system depends, partly, on the concentrations of cytokines produced by activated DC [41]. IL-12 and IL-6 produced by DC induce B cell differentiation and immunoglobulin production [38]. Thus, a reduction in IL-12 production by DC after DON exposure may play an important role in the observed effects on B cells.

The observed effects of DON on DC maturation and DC-dependent differentiation of T and B cells highlights the importance of studying the exposure to DON in early stages of life, as neonatal DC are functionally immature, with a lower capacity of IL-12 and IFN-γ production and a lower efficiency in antigen presentation, compared to adult DC [42]. These differences in DC signaling may contribute to a Th2-skewed status and reduced immune responsiveness in neonates [43]. Thus, a fully developed DC signaling is a prerequisite for the establishment of an efficient adaptive immune system in later stages of life [42]. Exposure to DON in the early stages of life, for example via maternal diet during pregnancy and lactation, may hinder the development of fully functional DC, leading to an imbalanced adaptive immune response in later stages of life.

Non-digestible oligosaccharides to prevent DON-induced immune modulations

Understanding the relation between mycotoxin exposure during pregnancy and immune-related health problems may provide a basis for developing nutritional interventions that can effectively prevent or attenuate these adverse effects at least in part. A potential approach for restoring the immunotoxicity of DON is dietary supplementation with non-digestible oligosaccharides (NDOs). Some specific NDOs have prebiotic and immune-modulating properties comparable to those observed for human milk oligosaccharides (HMOs). HMOs are crucial in the development of a healthy immune system in infants [44]. Some functional HMOs are based on elongation of lactose, forming different galactosyl-lactoses (GLs) such as 3'-GL, 4'-GL and 6'-GL [45-47]. There are several NDO mixtures currently available, such as galacto-oligosaccharides (scGOS), fructo-oligosaccharides (FOS) and trans-galacto-oligosaccharides (TOS), which are mainly manufactured by free enzymatic trans-glycosylation or through bacterial fermentation [48], and contain GLs comparable to those isolated from HMOs [49]. Although NDOs, as well as HMOs, are only partially digested by bacteria in the intestine [50, 51] some specific structures such as 2'-fucosyllactose (2'FL) and (GOS) are detectable in the systemic circulation after oral administration [52, 53].

Different mechanisms can explain the immunomodulatory properties of NDOs and HMOs. They are effective prebiotic ingredients and can induce immunomodulatory

effects indirectly by contributing to the development of a healthy intestinal microbial community in infants [54-56]. Moreover, HMOs induce microbiota-independent immunomodulatory effects through direct interaction with intestinal epithelial cells and immune competent cells [57, 58]. Dietary intervention with scGOS/lcFOS-containing NDO mixtures [57, 59, 60], as well as human milk oligosaccharide 2'FL [61], and their combinations [62], has been shown to improve Th1-dependent responses to Influenza vaccination.

To investigate whether specific HMOs have beneficial properties that would attenuate DON-induced immunotoxicity, TOS-supplemented diets were used in the vaccination model (**chapter 2**). TOS is a mixture of NDOs which contain high levels of 3'-GL. In the absence of DON dietary supplementation with 0.5% TOS enhanced Th1-dependent cellular and humoral immunity in vaccinated mice, demonstrated by an increased IgG2a production, a higher percentage of activated $CD69^+$ Th1 cells in the spleen and an increase in production of IFN-γ by re-stimulated splenocytes (**chapter 2**). However, these effects were not observed in vaccinated mice receiving a diet supplemented with 1% TOS, indicating that an optimal level of TOS is required to induce such beneficial properties. In contrast, in mice which were exposed to DON, 1% TOS was required to counteract the adverse effects of DON and boost the immune response to vaccine antigens (**chapter 2**); Addition of 1% TOS in DON-contaminated diets increased the frequency of $Tbet^+$ Th1 cells and secretion of IFN-γ from re-stimulated splenocytes and restored the DON-induced reduction in B cells and hence prevented the DON-induced reduction in type-1 immune responses in vaccinated animals. This apparent dose-dependency has been observed previously in studies using other types of specific NDOs in this experimental animal model. Dietary supplementation with 2'-FL up to 1% increased influenza-specific DTH response in vaccinated mice dose-dependently [61].

A possible mechanism to explain the positive effects of TOS on restoring Th1/Th2 balance in DON exposed animals could be an increase in regulatory immune response, as regulatory T cells are proven to play substantial role in vaccination responsiveness [57, 63]. Although DON exposure had no significant effect on regulatory T cell population in the spleen, TOS supplementation caused a significant increase in $FoxP3^+$ regulatory T cells in DON-exposed groups, and inhibited DON-induced reduction in IL-10 production (**chapter 2**).

Immunomodulatory properties of TOS may be linked to microbiota-dependent mechanisms [58]. There is a positive association between changes in gut microbial metabolites and improved vaccine-specific immune responses upon HMOS supplementation [62, 64]. In **chapter 2**, it was demonstrated that DON-contaminated diets significantly reduced SCFA concentrations in cecum content of mice, which was neutralized by TOS. Beneficial *Bifidobacteria* strains in gut are able to utilize 3'-GL content of TOS efficiently [65], hence the prebiotic properties of TOS possibly play a

role in modulating the immune responses. SCFA produced by gut bacteria, have well established anti-inflammatory properties, and regulate innate immune cells such as macrophages, neutrophils, and DC, as well as antigen-specific adaptive immunity mediated by T cells and B cells [66]. Moreover, NDOs can exert direct, microbiota-independent effects on immune cells [67], through binding with TLR in monocytes, macrophages, and intestinal epithelial cells [68, 69]. High levels of 3'-GL present in TOS may partly be responsible for the observed effects of TOS on the intestine, as both TOS and 3'-GL were able to protect intestinal epithelial integrity and prevent inflammatory responses induced by DON on Caco-2 cells *in vitro* (**chapter 2**). Therefore, dietary supplementation of infant formulae with TOS can be beneficial for boosting the immune responses and preventing harmful effects of DON on the immune system.

Part 2: Mycotoxins during pregnancy: adverse effects on placenta and fetus

Maternal nutrition during pregnancy and lactation directly defines fetal and infant nutrition and plays a crucial role in maintaining a healthy pregnancy and long-term health of the infant [70, 71]. The importance of a well-balanced maternal diet has been studied extensively [72, 73]. However, exposure to toxic food contaminants such as mycotoxins during pregnancy and/or lactation can be equally influential as it may lead to detrimental health effects for both the mother and the developing child. Mycotoxins such as DON, ZEN and T-2 toxin can pass through the placenta [74-76] and adversely affect the development of the fetus during pregnancy. These mycotoxins are detected in fetal and placental samples of pregnant sows and rats receiving mycotoxin-contaminated diets during pregnancy [75, 77-80]. Fetal death, brain damage [81-83], skeletal malformations [84], thymus atrophy [85], and reproductive problems in the offspring are observed upon maternal exposure to these Fusarium mycotoxins during pregnancy.

During pregnancy, the placenta is the only link between the fetus and mother, mediating maternal-fetal transfer of nutrients and metabolic waste products [86]. The placenta forms a barrier against pathogens and paracellular diffusion of chemicals and toxins [87]. In this thesis, the effect of DON, ZEN and T-2 toxin on the placental barrier was studied using BeWo cells, mimicking a placental cytotrophoblast cell layer (**Chapter 4**). This study was the first to report that DON and T2-toxin can increase paracellular permeability in BeWo cells by inducing significant changes in expression and localization of junctional proteins. The effect of ZEN on the barrier parameters was less prominent, compared to DON and T-2 toxin.

The effect of DON on placental barrier integrity was further confirmed *in vivo*, as consuming DON-contaminated diets in mice during pregnancy modified the mRNA and protein levels of junctional proteins in the placenta (**chapter 5**). Such disruption in

the integrity of placental barrier layer can potentially lead to imbalanced maternal-fetal transportation of nutrients and hazardous chemicals.

DON exposure in pregnant mice is shown to affect pregnancy and fetal development negatively [88, 89]. In **chapter 5** of this thesis, these negative effects of dietary DON exposure during pregnancy were confirmed on breeding success and fetal survival and detected a significant concentration of DON in the fetal compartment of pregnant DON-fed mice, which is consistent with earlier observations that DON can pass the placenta [77, 90, 91]. Direct exposure of the fetus to DON may partly account for its developmental toxicities such as skeletal deformities [88, 92]. In line with this, we observed several visible skeletal deformities in the tails of 40% of male offspring born to DON-exposed mice (**figure 1**). This effect of DON may be sex-dependent, as no obvious deformities were detected in the tails of the female offspring of DON-exposed mice. Understanding the mechanisms involved in these skeletal malformations requires further investigation, but the increased susceptibility of the males to such teratogenic effects might be due to a mutation on the mouse X chromosome that produces tail deformities which is not silenced in males [93].

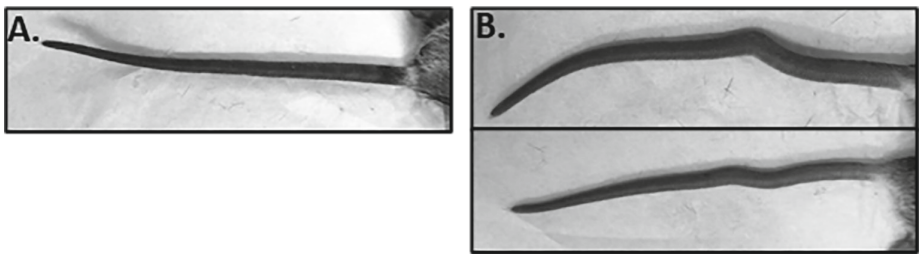


Figure 1. Skeletal deformity in male offspring, due to maternal deoxynivalenol (DON) exposure. Female C3H mice received either control or DON-contaminated diets (12.5 mg/kg of diet) throughout the pregnancy and lactation period. **A)** the tail of a male offspring born to control-fed mouse, **B)** the tails of male offspring born to mice fed DON-contaminated diet. The pictures are taken 6 weeks after birth.

In addition to its barrier function and regulating maternal-fetal transportation, the placenta plays an important role in responding to a great diversity of inflammatory stimuli [94]. A healthy pregnancy and development of the fetus requires a regulated immune response and specific cytokine milieu in the placenta. In **chapter 5**, it was demonstrated that DON exposure during pregnancy significantly altered the cytokine profile in the placenta and amniotic fluid. This is of importance as alterations in the immunological status of placenta is linked to a variety of pregnancy complications such as pre-eclampsia and miscarriage [95]. Excessive and imbalanced activation of immune cells in the placenta, especially T cells and NK cells, can cause pregnancy loss through the induction of pro-inflammatory responses [96]. Furthermore, an elevated production of inflammatory cytokines and a reduction in regulatory cytokines can exert detrimental effects in the placenta and induce abortion or preterm labor [97-99]. It could thus

be suggested that changes in the immune status of the placenta could play a part in inducing the observed low fetal survival rate in DON-exposed mice.

During a healthy pregnancy, the maternal immune system shifts towards a more tolerogenic state by downregulating Th1-mediated immune responses and increasing the production of regulatory and Th2-mediated cytokines in order to prevent rejection of the semi-allogeneic fetus [100-102]. In **chapter 5**, a slight decrease in the Th2 cell population and a shift in the Th1/Th2 balance towards Th1 in the spleen and iliac lymph nodes of pregnant mice receiving DON-contaminated diets was demonstrated. The maternal immune system is normally intricately regulated to maintain a healthy pregnancy. Thus, the observed changes in the maternal Th1/Th2/Treg balance, stress out the importance of further investigation into the DON-induced changes on immune parameters, especially in the placenta and maternal-fetal interface.

Part 3: Early-life DON exposure and immune programming

The most critical immune maturational events occur during the early stages of life [103, 104]. Therefore, any immune disturbance in this period can result in altered immune function and can even have significant long-term consequences for the offspring [105, 106]. Considering the immunotoxicity (**chapter 2 and 3**) and placental transfer of DON during pregnancy (**chapter 4 and 5**), the potential long-term immune modulations in the offspring caused by maternal DON exposure during pregnancy and lactation was studied (**chapter 6**).

Although a previous study reported no macroscopic lesions or detectable pathomorphological changes in spleen, lymph nodes, gut, and bone marrow of the fetuses from pregnant sows receiving a diet containing 4.42 mg/kg DON [107], intravenous administration of DON in pregnant pigs at the end of gestation altered mRNA expression of different cytokines such as IFN-γ, IL-17, IL-2, and TNF-α, in the blood leukocytes of the piglets, and induce significant decrease in the population of T cells 1-3 weeks after birth [91]. The changes in immune profile of the offspring indicate a disturbed and unbalanced immune system after birth due to maternal DON exposure. However, information regarding the long-term impact of exposure to DON during pregnancy on the immune system is scarce. In **chapter 6,** the effects of maternal DON exposure on the development of ovalbumin (OVA)-specific food allergy and antigen-specific immune responses to influenza vaccination in mice was studied. We showed for the first time that early-life DON exposure skewed the immune system in the offspring toward an imbalanced state, resulting in an increased allergic immune response to food allergens and a decreased responsiveness to vaccination against influenza virus. The observed effects can, partly, be due to the suppressed Th1-mediated immune capacity and Th1/Th2 imbalance towards Th2 in these animals.

Sensitizing female offspring to OVA using cholera toxin (CT) as an adjuvant caused stronger allergic responses in animals born to DON-exposed mice, as measured by increased acute allergic skin reactions (ASR) and more severe anaphylactic shock symptoms upon intradermal challenge with OVA. Concentrations of OVA-specific serum IgGs and IgE were higher in OVA+CT-treated offspring born to DON-exposed mice, than those born to control mice. Disrupted intestinal epithelial integrity could be a potential mechanism causing higher susceptibility to develop allergy after direct exposure to DON [108]. However, in our study, maternal DON exposure had no significant effect on mRNA expression of tight junctions in the small intestine of allergic mice (**chapter 6**). Similarly, as reported in **chapter 5**, no significant changes in mRNA expression of tight junction proteins were detected in fetal intestine due to DON exposure during pregnancy. These observations indicate that mechanisms other than DON-induced intestinal damage are involved in elevated allergic reactions in the offspring of DON-exposed mice. Analyzing the splenocytes collected from female offspring revealed no significant effect of maternal DON exposure on the population of splenic Th2 cells and the production of Th2-mediated cytokines, such as IL-13 and IL-4, after *ex vivo* re-stimulation. Interestingly, we observed significant reductions in $Tbet^+$ Th1 cell population and downregulation of Th1-mediated IFN-γ production in the female offspring of DON-exposed mothers. Reduction of Tbet expression is associated with the development of allergy in mice [109], mainly by disturbing the balance between Th1- and Th2-mediated immunity and shifting the balance toward Th2-mediated responses. This decrease in Th1 immunity may be involved in the observed increase of allergic responses in the female offspring.

Similarly, a significant reduction in the percentage of $Tbet^+$ Th1 cells in the spleen and the production of Th1-mediated cytokines, such as IFN-γ, IL-12p70 and IL-27, were observed in male offspring born to DON-exposed mice which received Influvac injections. A suppressed immune response to Influenza virus antigens was observed in these mice, indicated by a reduced DTH response and serum levels of antigen-specific IgGs after intradermal challenge. Th1-mediated cytokines, such as IFN-γ, play a key role in anti-microbial immunity and a decrease in the capacity of IFN-γ production can reduce host resistance to pathogens [110]. Similar effects on vaccination responsiveness and Th1-mediated immune responses after direct dietary exposure to DON were observed in **chapter 2**. Moreover, in line with the observations in **chapter 2 and 6**, a diminished Th1-polarizing capacity of DCs after DON exposure was reported in **chapter 3**. Overall, it can be concluded that DON exposure, directly or indirectly via the maternal diet, can lead to an imbalanced Th1/Th2 immune response and hence to a shift toward higher Th2-associated immunity.

Although direct exposure to DON revealed no significant effect on $FoxP3^+$ regulatory T cells in vaccinated mice (**chapter 2**), a downregulation of $Foxp3^+$ regulatory T cells was observed in the spleens of both allergic and vaccinated mice born to DON-exposed mothers (**chapter 6**). This indicates that a reduction in regulatory T cells may occur

due to the exposure to DON during the early stages of immune development or as a consequence of indirect exposure to DON, via maternal diet during pregnancy. Regulatory T cells have a prominent role in vaccination responsiveness [57, 63] and allergy development [111]. Treg populations actively prevent hypersensitive reactions by suppressing Th2 responses [112, 113]. Therefore, the reduction in the Treg cell population may have contributed to the imbalanced immune responses observed in the offspring of DON-exposed mice.

Interestingly, exposing female offspring of DON-exposed mice to OVA without CT also induced an increase in ASR and production of serum IgGs after OVA challenge, although OVA-specific IgE levels were not affected (**chapter 6**). These observations indicate that early-life DON exposure can facilitate allergy development in the absence of an adjuvant, possibly through potential non-IgE-mediated mechanisms. Similarly, a reduction in $Tbet^+$ Th1 cells and Th1-mediated cytokines was observed in these mice which may partly explain the increased ASR and vaccine-specific IgG production in these animals. Nevertheless, larger sample size for OVA-treated group is required for definite conclusion.

Overall, it can be concluded that early-life dietary exposure to the food contaminant DON can adversely influence immune maturation and development in the offspring. Consequently, the immune system in the offspring is skewed toward an imbalanced state. Further investigation is required to fully understand the exact mechanisms involved in DON-induced developmental immunotoxicity.

Conclusion and future perspective

The main findings of this thesis are summarized in **figure 2**. In this thesis, we showed that exposure to the major Fusarium mycotoxin DON, can adversely affect innate and adaptive immune responses, placental barrier, fetal survival and development. We further demonstrated that consuming DON-contaminated diets during pregnancy and lactation, induces long-term effects on the developing immune system of the offspring and shifts the balance in immune cell populations, hence increasing the susceptibility to allergic reactions and reducing immune responses to vaccination in later stages of life.

Moreover, we showed that nutritional supplementation with a specific NDO mixture rich in 3'-GL, can be beneficial in preventing or neutralizing the adverse effects of DON on immune system. Thus, NDOs might be a suitable candidate as a supplement in infant formulae for boosting the immune system and preventing the harmful effects of early-life exposure to DON on the intestinal and immune systems; however, further investigation is required to unravel the exact mechanism of the protective effects of NDOs against DON-induced immunotoxicity.

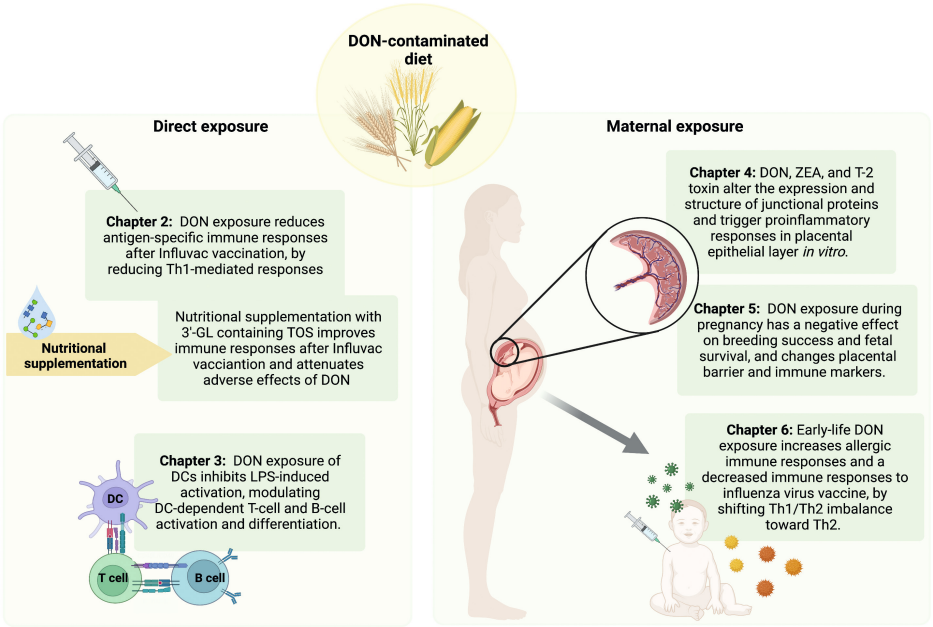


Figure 2. A schematic summary of the main outcomes in each chapter of this thesis. This figure is created with BioRender.com.

In this thesis we focused on continued exposure to DON in early-life through diet, as in physiological conditions the exposure may occur constantly throughout the entire period of pregnancy and lactation, depending on the maternal diet. However, further studying the effect of DON exposure at different developmental stages separately, such as first, second and third trimesters of pregnancy and lactation, would help to determine the most critical window for DON-induced immunotoxicities.

Although the concentrations of DON used in this thesis (12.5 mg/kg) were higher than the maximum permitted levels set by the European Commission (1-2 mg/kg of food) [114], analyzing cereal samples from all over the world revealed a considerable percentage of food products exceeding this safety level [115-117], as DON concentration in several commodities were reported to be as high as 20 mg/kg of food [118]. Moreover, combined consumption of a wide variety of DON-contaminated food can lead to a significantly higher exposure levels in specific populations. In addition, masked and modified forms of DON, which are not detected by established standard methods, may lead to underreporting of mycotoxin levels [119]. Thus, investigating the toxicity of DON at a wide range of concentrations, as well as in combination with its acetylated modified forms, is of utmost importance.

In this thesis a special focus was placed on DON, as the most prevalently occurring food contaminant. However, co-contamination of food products with multiple mycotoxins occurs frequently, since many fungi species are able to produce several mycotoxins simultaneously [120]. Thus, humans are generally exposed to several toxins at the same time [121]. Therefore, further investigations on the toxicity of a combination of commonly co-occurring mycotoxins, will provide a better understanding of the potential additive or synergistic effects of these food contaminants on human health.

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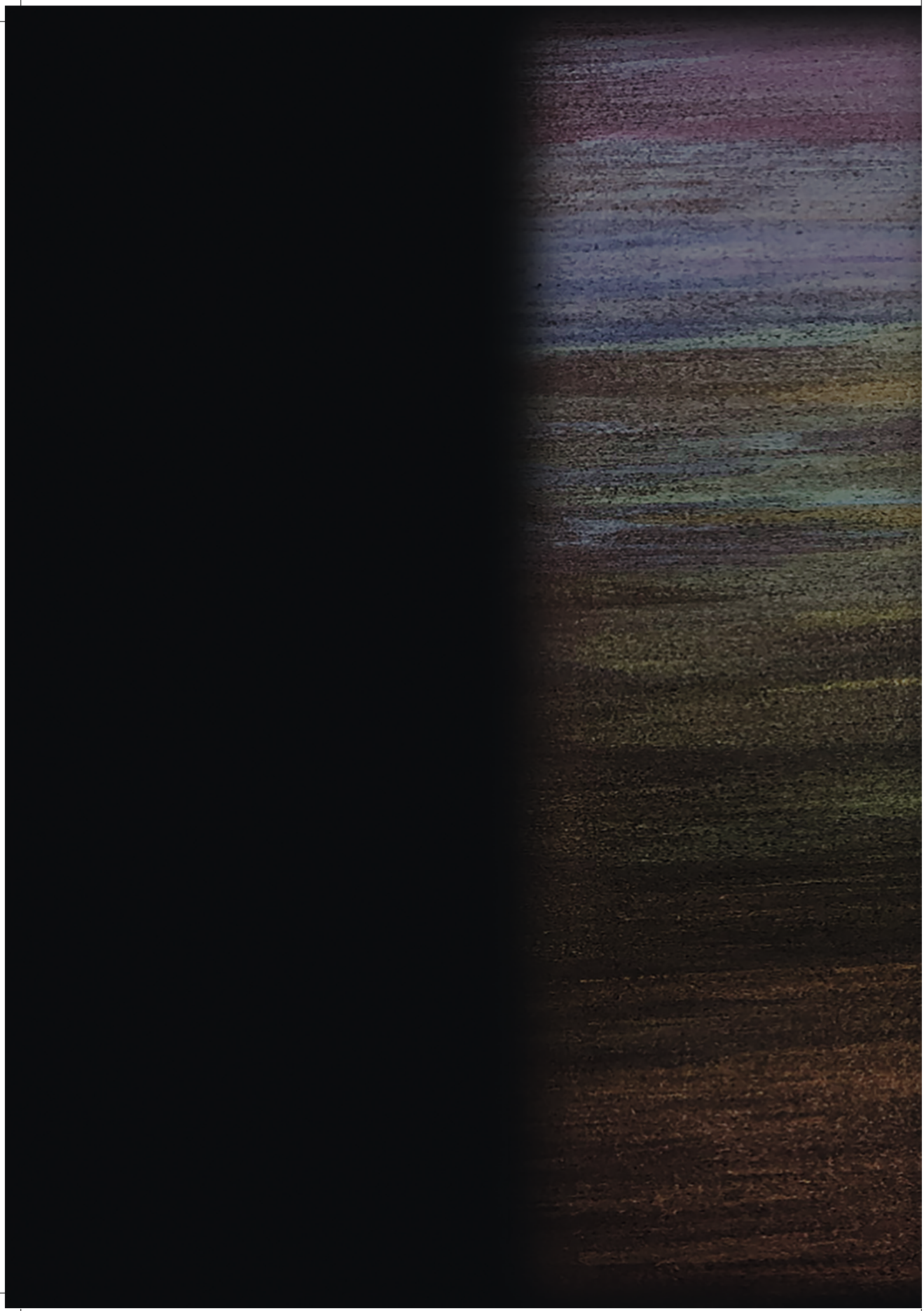

Appendices

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English summary

Mycotoxins are among the most important and highly prevalent nutritional contaminants worldwide. They are naturally produced as secondary metabolites of different fungal species, which can contaminate a wide range of agricultural products, especially cereal and grain-based food. Fusarium species are amongst the most prevalent fungal contaminants of grains, and produce the two most important classes of mycotoxins, trichothecenes (including deoxynivalenol (DON), T-2 toxin and HT-2 toxin) and zearalenones (ZEN). In humans and animals, exposure to Fusarium mycotoxins is associated with mutagenic, teratogenic, estrogenic, hemorrhagic, neurotoxic, hepatotoxic, and immunotoxic effects. In **chapter 1** the toxicities of DON, T-2 toxin and ZEN on main biological systems and organs, especially the immune and reproductive systems, as well as on the developing embryo and fetus are summarized.

Considering the widespread occurrence of these food contaminants and inevitable human exposure, this thesis aimed to investigate the adverse effects of exposure to commonly occurring Fusarium mycotoxins and potential mechanisms involved, with a special focus on DON, as this is the most prevalently occurring food contaminant worldwide. **Part 1** of this thesis (**chapter 2** and **3**) focused on the adverse effects of DON on the innate and adaptive immune responses and possible mechanisms and pathways involved. In **part 2** (**chapter 4** and **5**), the effects of DON exposure on pregnancy, fetal survival and placental structure and function were investigated. Finally, in **part 3** (**chapter 6**), the long-term impact of early-life DON exposure on the immune development was studied.

The immune system is highly sensitive to Fusarium mycotoxins and exposure to low levels that do not induce explicit acute toxicity, significantly modulate immune responses. In **chapter 2,** the effects of DON exposure on antigen-specific adaptive cellular and humoral immune responses are demonstrated, with a focus on Th1-mediated immunity, using a validated murine vaccination model. Exposure to DON-contaminated diets downregulated immune responses to vaccination through reducing Th1-mediated cellular and humoral immune responses.

Some specific non-digestible oligosaccharides (NDOs) have prebiotic and immune-modulating properties comparable to those observed for certain human milk oligosaccharides (HMOs). Specific structures of NDOs and HMOs are confirmed to have positive effects on improving adaptive immune responses. Therefore, **in chapter 2** the possible immunomodulatory effects of dietary supplementation with a specific mixture of NDOs, trans-galactosyl-oligosaccharides (TOS), on the vaccine responsiveness in DON-exposed mice was investigated. Dietary intervention with TOS, attenuated the adverse effects of DON on the systemic adaptive immune response by restoring the Th1/Th2 balance and improving vaccine responsiveness. The main oligosaccharide

component of TOS is 3'-galactosyl-lactose (3'-GL), which is comparable to the specific oligosaccharide structures present in human milk. Therefore, an *in vitro* model of the intestinal epithelial cell layer was used in **chapter 2** to understand the possible mechanisms involved in DON- and 3'-GL-induced changes on the barrier and immune function of intestine. The effects of TOS on the epithelial barrier are possibly linked to the 3'-GL present in the mixture. Therefore, dietary supplementation of infant formulae with TOS could be beneficial in boosting the immune system and preventing the harmful effects of food-contaminant DON on the intestinal and immune systems.

In **chapter 3**, an *in vitro* allogeneic coculture model was introduced to study the interplay between human monocyte-derived dendritic cells (DC), naïve T cells and B cells, and this model is used to investigate the effects of DON exposure on DC maturation and activation, as well as on DC-dependent activation and differentiation of naïve T cells and B cells. DON exposure of stimulated DC diminished the capacity of these cells to activate T cells and/or induce Th1-polarization, hence skewing T cell responses toward Th2. Moreover, DON exposure inhibited DC- and T cell-dependent B cell differentiation to plasma cells and IgM production in a triple DC-T cell- B cell coculture system.

Pregnancy is a critical period of physiological changes for both the mother and the fetus and maternal nutrition plays a crucial role during this period to shape the long-term health of the infant. During pregnancy, the placenta is the only link between the fetus and the mother. It is important as a barrier against pathogens and paracellular diffusion of chemicals and toxins. Any disruption of the integrity of this layer leads to imbalanced maternal–fetal transportation of nutrients and hazardous chemicals. DON, T-2 toxin and ZEN can pass through the placenta during pregnancy. This indicates that the developing fetus can potentially be at risk of mycotoxin-induced adverse effects. In **chapter 4**, the effects of exposure to DON, T-2 toxin and ZEN were investigated in BeWo cells which serve as an *in vitro* model of the human placental epithelial cell layer. By evaluating the expression and localization of junctional proteins and the expression of pro-inflammatory cytokines, the effects of mycotoxin exposure on placental barrier function and inflammatory responses were assessed. Results of this study revealed an increased paracellular permeability in BeWo cells after mycotoxin exposure, mainly by inducing significant changes in expression and localization of junctional proteins.

In **chapter 5**, the consequences of chronic exposure to dietary DON on pregnancy were assessed in a murine model. Changes in the placental barrier and immune markers, as well as the immune modulations in the mother and the fetus were investigated. This study confirmed the negative effects of DON-contaminated diets on breeding success and fetal survival in mice, accompanied by altered mRNA and protein levels of junctional proteins in the placenta. In addition to its barrier function and regulating maternal-fetal transportation, the placenta plays an important role in responding to a great diversity of inflammatory stimuli. Results of the study in **chapter 5** showed that DON exposure

during pregnancy significantly altered the cytokine profile in the placenta, as well as in the amniotic fluid.

The most critical events in immune development and maturation occur early in life, especially during pregnancy and lactation periods. Therefore, any immune disturbance at this stage can impose significant long-term consequences for the offspring. Considering the potential DON-induced immune modulations, in **chapter 6** the effects of maternal exposure to DON during pregnancy and lactation on the immune system of the offspring were investigated. The murine model of ovalbumin (OVA)-induced food allergy was used to examine the Th2-mediated allergic response and the influenza-vaccination model was used to assess the Th1-mediated vaccination responses in the offspring. Early-life DON exposure skewed the immune system in the offspring towards an imbalanced state, resulting in an increased allergic immune response and a decreased responsiveness to influenza virus vaccination. The observed effects could partly be due to the diminished Th1-mediated immune capacity and Th1/Th2 imbalance towards Th2 in these animals.

Finally, in **chapter 7** the main findings of this thesis and the overall conclusions are discussed. Briefly, exposure to the major Fusarium mycotoxin, DON, adversely affects innate and adaptive immune responses and DC/T-cell/B-cell interactions, pregnancy, and fetal development, as well as the placental barrier and immune function. Furthermore, consuming DON-contaminated diets during pregnancy and lactation, induces long-term effects on the developing immune system of the offspring, leading to an increased susceptibility to allergic reactions and reduced immune responses to vaccination. Finally, nutritional interventions with specific NDOs containing 3'-GL were proposed to be beneficial for attenuating and restoring the adverse effects of DON on the immune system.

Nederlandse samenvatting

Mycotoxinen behoren tot de belangrijkste en meest voorkomende voedingscontaminanten ter wereld en zijn secundaire metabolieten die van nature geproduceerd worden door verschillende schimmelsoorten. Deze schimmels kunnen een breed scala aan landbouwproducten besmetten, met name granen en voedsel op basis van granen. Fusarium-schimmels zijn een van de meest voorkomende schimmels op granen. Deze Fusarium-schimmels kunnen twee belangrijke klassen van mycotoxinen produceren, te weten de trichothecenen (inclusief deoxynivalenol (DON), T-2-toxine en HT-2-toxine) en de zearalenonen (ZEN). Zowel bij mensen als bij dieren wordt blootstelling aan Fusarium-mycotoxinen in verband gebracht met mutagene, teratogene, oestrogene, hemorragische, neurotoxische, hepatotoxische en immunotoxische effecten. In **hoofdstuk 1** is informatie verzameld over de toxische effecten van DON, T-2-toxine en ZEN op de belangrijkste biologische systemen en organen, in het bijzonder het immuunsysteem. Tevens zijn toxische effecten van deze myxotoxinen op de ontwikkeling van embryo's en foetussen beschreven.

Deze mycotoxinen kunnen makkelijk worden verspreid en zorgen voor een onvermijdelijke humane blootstelling. Dit proefschrift is gericht op het onderzoeken van de nadelige effecten van blootstelling aan veel voorkomende Fusarium-mycotoxinen en de mogelijke mechanismen die daarbij betrokken zijn. Hierbij werd speciale aandacht besteed aan DON, aangezien dit wereldwijd de meest voorkomende voedselcontaminant is. **Deel 1** van dit proefschrift (**hoofdstuk 2 en 3**) is gewijd aan de nadelige effecten van DON op de aangeboren en adaptieve afweerreactie en mogelijke mechanismen en routes die daarbij betrokken zijn. In **deel 2** (**hoofdstuk 4 en 5**) worden de effecten van blootstelling aan DON op de zwangerschap, de overleving van de foetus en de structuur en functie van de placenta onderzocht. Ten slotte wordt in **deel 3** (**hoofdstuk 6**) de blootstelling van DON tijdens de zwangerschap en lactatie, op de ontwikkeling van het afweersysteem van de nakomelingen op langere termijn onderzocht.

Het immuunsysteem is zeer gevoelig voor Fusarium-mycotoxinen; blootstelling aan lage doseringen (concentraties die geen expliciete acute toxiciteit induceren), kunnen afweerreactie nadelig beïnvloeden. In **hoofdstuk 2** hebben we de effecten van blootstelling aan DON op de antigeenspecifieke cellulaire en humorale adaptieve immuunresponsenafweerreactie aangetoond in een gevalideerd vaccinatiemodel in de muis. De resultaten van deze studie toonden aan dat blootstelling aan diëten besmet met DON, de afweerreactie na vaccinatie verlaagde door de Th1-gemedieerde cellulaire en humorale immuunrespons bij muizen te verminderen.

Specifieke niet-verteerbare oligosachariden (NDOs), hebben prebiotische en immuunmodulerende eigenschappen die vergelijkbaar zijn eigenschappen van bepaalde oligosachariden in moedermelk (HMOs). Van specifieke structuren van NDOs en HMOs

is bevestigd dat ze positieve effecten hebben op het verbeteren van de adaptieve afweerreactie. Daarom worden in **hoofdstuk 2** de mogelijke immunomodulerende effecten op de vaccinrespons in DON-blootgestelde muizen onderzocht nadat ze een specifiek mengsel van NDOs, trans-galactosyl-oligosacchariden (TOS) hadden gekregen. Dieetinterventie met TOS verminderde de nadelige effecten van DON op de systemische adaptieve afweerreactie door de Th1/Th2-balans te herstellen en de afweer na vaccinatie te verbeteren. De belangrijkste oligosacharidecomponent van TOS is 3'-galactosyl-lactose (3'-GL) en deze structuur is vergelijkbaar met specifieke oligosacharidestructuren die aanwezig zijn in moedermelk. Om deze reden is met behulp van een *in vitro* model met darmepitheelcellen (**hoofdstuk 2)** de mogelijke mechanismen bestudeerd, die betrokken zijn bij DON- en 3'-GL-geïnduceerde veranderingen in de darmbarrière en het afweersysteem in de darm. De resultaten van dit experiment laten zien dat de effecten van TOS op de darmepitheelbarrière mogelijk verband houden met de 3'-GL, dat in het mengsel aanwezig is. Daarom zou het toevoegen van TOS aan zuigelingenvoeding mogelijk gunstig kunnen zijn voor het stimuleren van het afweersysteem en het voorkomen van de schadelijke effecten van voedselverontreiniging met DON op de darm en het immuunsysteem.

In **hoofdstuk 3** wordt een *in vitro* model geïntroduceerd om het samenspel tussen verschillende humane cellen van het afweersysteem, dendritische cellen (DCs), naïeve T-cellen en B-cellen, te bestuderen. Dit model wordt gebruikt om de effecten van DON-blootstelling op de rijping en activering te bestuderen van DCs , evenals de DC-afhankelijke activering en differentiatie van naïeve T-cellen en B-cellen, te onderzoeken. De resultaten van deze studie toonden aan dat gestimuleerde DCs blootsteld aan DON, het vermogen van deze cellen om T-cellen te activeren reduceerde en ook hun capaciteit om Th1-polarisatie te induceren verminderde. Hierdoor werden de T-cell responsen naar een Th2 profiel-gemedieerde afweerreactie verschoven. Daarnaast konden we middels het drievoudig DC-T-cel-B-cel-cocultuursysteem aantonen dat DON-blootstelling, de DC- en T-celafhankelijke B-cel differentiatie tot plasmacellen remde en was er een lagere productie van IgM.

Zwangerschap is een kritieke periode van fysiologische veranderingen voor zowel de moeder als de foetus. De voeding van de moeder speelt een cruciale rol in deze periode en biedt kans om de gezondheid van het kind op de lange termijn te waarborgen. Tijdens de zwangerschap is de placenta de enige schakel tussen de foetus en de moeder. De placenta is belangrijk als een barrière tegen het binnendringen van pathogenen chemicaliën en toxines. Daarom leidt elke verstoring van deze mogelijk barrière tot problemen in het transport van voedingsstoffen tussen moeder en foetus en het binnendringen van pathogenen en andere schadelijke stoffen. Van DON, T-2-toxine en ZEN is bekend dat ze tijdens de zwangerschap de placenta kunnen passeren. Dit geeft aan dat de ontwikkelende foetus mogelijk risico loopt op schadelijke effecten veroorzaakt door deze mycotoxinen effecten. In **hoofdstuk 4** worden de effecten van

blootstelling aan DON, T-2 toxine en ZEN onderzocht in BeWo-cellen die dienen als een geschikt *in vitro* model voor het nabootsen van de humane epitheelcellaag van de placenta. Door de expressie en lokalisatie van junctionele eiwitten (eiwitten die belangrijk zijn voor een sterke barrière) en de uitscheiding van ontstekingseiwitten (cytokines) te bestuderen, werden de effecten van blootstelling aan mycotoxinen op de barrièrefunctie en de afweerreactie van de placenta onderzocht. Resultaten van deze studie lieten een verhoogde permeabiliteit in BeWo-cellen zien na blootstelling aan mycotoxinen, voornamelijk door significante veranderingen in expressie en lokalisatie van junctionele eiwitten.

In **hoofdstuk 5** worden de gevolgen van chronische blootstelling aan DON via voeding op de zwangerschap onderzocht in muizen. Veranderingen in de barrière van de placenta en in de afweerreacties van de moeder en de foetus werden onderzocht. Deze studie bevestigde de negatieve effecten van diëten besmet met DON op het foksucces. Tevens werden veranderingen gezien op de expressie van junctionele eiwitten in de placenta. Naast de barrièrefunctie en het reguleren van het maternale-foetale transport, speelt de placenta een belangrijke rol bij het reageren op diverse stimuli voor ontsteking. Bovendien werd aangetoond dat blootstelling aan DON tijdens de zwangerschap, het cytokineprofiel in de placenta en in het vruchtwater veranderde.

De meest kritieke gebeurtenissen in de ontwikkeling van het afweersysteem vinden vroeg in het leven plaats, vooral tijdens zwangerschap en de lactatieperiode. Daarom kan elke verstoring in immuunsysteem in dit stadium aanzienlijke langetermijngevolgen hebben voor het nageslacht. Gezien de mogelijke effecten van DON -op het afweersysteem, worden in **hoofdstuk 6** de effecten van maternale blootstelling aan DON tijdens zwangerschap en lactatie op het afweersysteem van het nageslacht onderzocht. Een muismodel voor voedselallergie werd gebruikt om de Th2-gemedieerde allergische reactie te onderzoeken, en het griepvaccinatiemodel werd gebruikt om de Th1-gemedieerde reactie op de vaccinatie bij de nakomelingen te beoordelen. De resultaten van deze studie toonden aan dat blootstelling aan DON op jonge leeftijd, het afweersysteem negatief beïnvloedt wat resulteert in een verhoogde allergische immuunreactie en een verminderde reactie op vaccinatie tegen het influenzavirus. De waargenomen effecten kunnen deels te wijten zijn aan de verminderde Th1-gemedieerde afweerreactie en verstoring van de Th1/Th2 balans.

Ten slotte worden in **hoofdstuk 7** de belangrijkste bevindingen en de algemene conclusies van dit proefschrift besproken. Samenvattend: in dit proefschrift wordt aangetoond dat blootstelling aan de belangrijkste Fusarium-mycotoxine DON een negatief effect kan hebben op aangeboren en adaptieve immuunreacties DC/T-cel/B-cel interacties, zwangerschap en foetale ontwikkeling, evenals op de barrière and afweersysteem van de placenta. Verder werd aangetoond dat het consumeren van voeding besmet met DON - tijdens zwangerschap en lactatie lange termijneffecten kan

hebben op het ontwikkelende afweersysteem van het nageslacht, wat leidt tot een verhoogde gevoeligheid voor allergische reacties en een verminderde afweerreactie na vaccinatie. Ten slotte werd voorgesteld dat voedingsinterventies met specifieke NDOs, die 3'-GL bevatten, gunstig zijn voor het verminderen en herstellen van de nadelige effecten van DON op het afweersysteem.

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Curriculum Vitae

Negisa Seyed Toutounchi (full name: Seyedehnegisa Seyedtoutounchi) was born on 5 August 1992 in Tabriz, Iran. After finishing high school in NODET (National Organization for Development of Exceptional Talents), she entered Tabriz University of Medical Sciences in 2010 to study pharmacy, and obtained her PharmD degree in April 2016. In 2012, she was ranked as top 2 students in the country in National Comprehensive Basic Sciences exam and became a member of National Elites Foundation in 2013. During her PharmD studies, she joined the research committee of Tabriz University and participated in several research projects in cardiovascular pharmacology lab, under supervision of Prof.Dr. Alireza Garjani. In the same lab, she worked on her final PharmD thesis about cardioprotective effects of rosmarinic acid on isoproterenol-induced myocardial infarction. In April 2017, Negisa started as a PhD candidate in pharmacology department of Utrecht University, in collaboration with Danone Nutricia Research and under supervision of Prof.Dr. Gert Folkerts, Prof.Dr. Johan Garssen, Dr. Astrid Hogenkamp, Dr. Saskia Braber and Dr. Belinda van 't Land. The focus of her PhD project was to investigate the effects of exposure to major Fusarium mycotoxin, deoxynivalenol, on pregnancy and immune development. The results of this project are compiled in this thesis. During her PhD studies, Negisa was actively participating in several international conferences, and she won travel grants from EAACI winter school 2020, WIRM 2020, and WIRM 2022. As of June 2022, Negisa has joined research group of Dr. Joke den Haan in Amsterdam UMC (VUmc) as a Postdoc researcher, to investigate on developing glycolipid liposomal vaccines to target tissue resident memory T cells in liver cancer.

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