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Abbreviations: ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; DON, deoxynivalenol; FCS, foetal calf serum; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; IL-8, interleukin-8; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MPO, myeloperoxidase; PMNs, polymorphonuclear cells; ROS, reactive oxygen species; TMB, 3,3',5,5'-Tetramethylbenzidine

Keywords: Apoptosis/ Chemotaxis/ Deoxynivalenol/ Phagocytosis/ Pig neutrophils

Abstract

Scope: Deoxynivalenol (DON), a mycotoxin produced by *Fusarium* spp., is toxic to many animal species, with pigs being the most sensitive species to the toxin. The aim of the present study was to determine the effects of DON on pig polymorphonuclear cells (PMNs), the first line of defence against infection.
Methods and results: PMNs isolated from pig blood samples were stimulated with LPS to mimic infection. DON (0.5 to 10 μM) altered three main functions of pig PMNs: LPS-induced secretion of IL-8, chemotaxis and phagocytosis capability. This alteration of PMN properties was due to apoptotis induced by DON exposure. Using Western blot and flow cytometry, we demonstrated that this process included the permeabilization of the mitochondrial outer membrane and the activation of caspase-3. The effect of DON was mediated by the phosphorylation of the p38 mitogen activated protein kinase (MAPK) within the first 30 min of exposure.
Conclusion: This study provides evidence that low concentrations of DON can alter the immune functions of porcine PMNs and suggests the involvement of p38 MAPK in the signal transduction pathway. These immunosuppressive effects of DON may have implications for humans and/or animals when eating contaminated food/feed.

1 Introduction:

Food safety is a major issue of concern for animal and public health in Europe. In this respect, much attention needs to be paid to the possible contamination of food by fungi and the risk of toxin production. Mycotoxins are secondary metabolites produced by fungi that may contaminate all stages of the food chain. Their occurrence world-wide is regarded as an important risk factor for human and animal health as up to 25% of the world crop production may be contaminated with mycotoxins [1]. The toxicological consequences of the ingestion of mycotoxins range from the induction of cancer and growth impairment to death [2]. The consumption of fungal toxins may also decrease resistance to infectious diseases [3].

Deoxynivalenol (DON) is a mycotoxin of the trichothecenes family that is mainly produced by *Fusarium graminearum* and *F. culmorum*. DON is commonly detected in cereals and grains, particularly in wheat, barley, maize and their by-products. It is the most prevalent contaminating mycotoxin in crop production in Europe and North America [4]. Furthermore, this toxin is resistant to milling, processing and heating and can therefore readily enter the food and feed chains [5].

DON is toxic to humans as well as to all animal species investigated so far [6]. Among animal species, pigs show high sensitivity to DON: acute exposure to a dose as low as 50µg/kg body weight can cause vomiting [7]. Through the high percentage of wheat in their diets, pigs could be frequently exposed to this toxin. Given that the gastrointestinal tract and the immune system of the pig are very similar to those of humans, the pig can be regarded as a model that can be applied to humans [8].

Acute exposure of pigs to high doses of DON induces radiomimetic effects including diarrhea, vomiting, leukocytosis and hemorrhages as well as necrosis in the gastrointestinal tract [7, 9]. Chronic toxicity studies have shown anorexia, reduced weight gain, altered nutritional efficiency and immunomodulation [6, 10]. At the cellular level, DON interacts with the peptidyltransferase at the 60S ribosomal subunit, triggering a translational arrest and activation of MAPK (p38, ERK, JNK) [11].

Immune cells are a primary target for DON, and depending on the dose and frequency of exposure, DON can be either immunosuppressive at high concentration or immunostimulatory at low concentration. The immunomodulatory effects of DON have been described on a macrophage cell line [12, 13], natural killer cells [14], lymphocytes [15], and dendritic cells [16] but very few studies have investigated the effect of DON on PMNs [17,18]. PMNs constitute the first line of defence against intruding microorganisms. Circulating PMNs have a short life-span at the end of which they undergo spontaneous apoptosis that leads to their clearance by macrophages. This process prevents secondary necrosis that would release injurious neutrophil contents into the surrounding tissue. In the case of infection, host factors such as cytokines and pathogen derived factors such as lipopolysaccharide (LPS) delay the apoptosis. Through chemotaxis, PMNs efficiently migrate to the inflammatory site where they phagocytose microorganisms and synthesize various inflammatory cytokines and chemokines such as IL-8 [19].

Using the pig model, the aim of the present study was to evaluate the effect of DON on several functions of LPS-activated PMNs, including phagocytosis, chemotaxis and IL-8 synthesis, and to determine the underlying mechanism. The presence of LPS in the current experiments was aimed at mimicking neutrophil stimulation triggered by Gram-negative bacteria infection and thus to investigate the capacity of DON to interfere with the innate immune system in response to a pathogen.

2 Materials and methods

2.1 Animals

Twelve crossbred pigs were used in this study, in accordance with the National Institute of Health Guidelines and those of the French Ministry of Agriculture for the Care and the Use of Laboratory Animals. They were housed in floored indoor pens in the animal facility of the INRA Research Center in Food Toxicology (Toxalim, Toulouse, France). The animals were kept two weeks to acclimatize prior to being used for blood collection at 6 to 12 weeks of age.

2.2 Blood sampling and neutrophil isolation

Blood samples were aseptically collected from the left jugular vein of pigs in sodium heparin tubes (Vacutainer®, Becton-Dickinson, USA). PMNs were isolated using a modification of a method previously described [20]. Briefly, 15 mL of the collected blood was diluted with an equal volume of HBSS. Five mL of diluted blood were gently layered over 6 mL of lymphocyte separation medium (Eurobio, Les Ulis, France) in 15 mL tubes and centrifuged at 800g for 30 min. The cell pellet was diluted in ice-cold hypotonic lysis solution (155 mM NH_4CL, 0.1 mM EDTA, 10 mM $KHCO_3$), and kept for 10 min. on ice to lyse the erythrocytes. After centrifugation, PMNs were washed once with HBSS solution and re-suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS (Eurobio, Les Ulis, France), 2mM L-glutamine, 100 U/mL penicillin and 50μg/mL streptomycin (Sigma-Aldrich, Saint–Quentin Fallavier, France). The proportion of PMNs as determined by microscopic observation after Giemsa coloration was greater than 95% and their viability was over 95% as assessed by trypan blue exclusion.

2.3 Determination of interleukin 8 (IL-8) concentration by ELISA

IL-8 content was measured using a sandwich ELISA implying two anti-porcine IL-8 antibodies. PMNs were stimulated or not with 10 µg/mL LPS (from *Escherichia coli* 0111:B4), and treated with 0 to 50 μM DON (Sigma-Aldrich, Saint–Quentin Fallavier, France) for 3 to 24 hours. Culture supernatants were then collected and the CXCL/IL-8 concentration was determined using a commercialized ELISA DuoSet® kit (R&D Systems, Lille, France) as previously described [21]. Briefly, the monoclonal anti-porcine CXCL/IL-8 (clone 105105) was used as the capture antibody in conjunction with polyclonal biotinylated goat anti-porcine IL8 antibody as detecting antibody. HRP and TMB were used for detection. Absorbance was read at 450 nm with an ELISA plate reader (Spectra Thermo Tecan, Trappes, France). Cytokine production was quantified by reference to standard curves constructed with known concentrations of recombinant porcine IL-8.

2.4 Chemotaxis assay

Chemotaxis was assessed by measuring the capacity of PMNs to move through a filter to a compartment containing a chemottractant. PMN migration was measured in Transwell inserts as previously described [22]. Briefly, 2 mL of PMNs (5 x 10^5 cells) were incubated at 39°C for 1 hour in serum-free DMEM, with increasing concentrations of DON (0 to 50 µM). 100 µL of cell suspension were then transferred into Chemocell® Transwell filters (Kurabo, Tokyo, Japan) placed in 24-well plates containing 5µg/mL recombinant porcine IL-8 in 450 µL HBSS acting as a chemoattractant. Supplementary wells were allocated for controls without chemoattractant. PMNs were allowed to migrate for 2h at 39°C and the number of migrated PMNs was measured by myeloperoxidase (MPO) activity. Transmigrated PMNs were lysed with 100 µL of lysis buffer (0.5% Triton X-100, 0.1mM citrate buffer, pH 4.2). MPO activity was assayed by reading the optical density at 405 nm, after addition of an equal volume of 0.1 mM citrate buffer containing 1 mM ABTS and 10 mM of H_2O_2. After appropriate color development, the reaction was stopped with 20% SDS (v:v). The number of

migrated PMNs was calculated from a standard curve established from known concentrations of the same PMN preparation.

2.5 Phagocytosis of BODIPY-labeled *Escherichia coli*

Phagocytosis ability was assessed by measuring the uptake of *E. coli* bacteria by PMNs. PMNs (5 x 10^5 cells) were cultured for 3 hours in 96-well plates in the presence of LPS (10 μg/mL) and increasing concentrations of DON. After DON exposure, the plates were centrifuged at 340 x g for 6 min at 5°C and the supernatant was discarded. Then, 100 μL of fresh DMEM medium containing 10% FCS and 5 x 10^6 BODIPY *E. coli* bioparticles (Molecular Probes, Madison, WI, USA) were added to each well. The resulting cell suspension was incubated for a further 45 min at 39°C, except the control, which was placed on ice for the same time. The plates were then centrifuged (340 x g for 6 min, 5°C) and washed twice with 200 μL HBSS. Finally, the cells were resuspended in 200 μL PBS-BSA 0.5% and 30 μL of a crystal violet solution (2 mg/mL) was added. After 15 min, BODIPY fluoresence was recorded using a flow cytometer. A phagocytic index was determined using the calculation: (MFI $_{\text{PMNs with DON}}$ - MFI $_{\text{PMNs on ice}}$) / (MFI $_{\text{PMNs without DON}}$ - MFI $_{\text{PMNs on ice}}$)

2.6 Assessment of apoptotic PMNs

Apoptotic cells were identified by detecting the translocation of phosphatidylserine to the outer leaflet of the cell membrane using AnnexinV-Alexa488. 10^6 PMNs were seeded in 24-well plates, stimulated with 10 µg/mL LPS and exposed to increasing concentrations of DON for 3 hours and 8 hours at 39°C. The cells were then harvested and stained with AnnexinV-Alexa488 and PI using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis assay (Molecular Probes, Madison, WI, USA). Briefly, Alexa488 staining was performed for 20 min at 5°C in the dark in 0.2 mL binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). To identify necrotic cells, 0.2 µg propidium iodide (PI) was added just before analysis. Alexa488 and PI fluorescence were recorded using a flow cytometer.

2.7 Measurement of the mitochondrial transmembrane potential (ΔΨm)

The mitochondrial transmembrane potential was assessed using the cationic fluorescent probe 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) as previously described [23]. $DiOC_6$ diffuses through the plasma membrane and accumulates inside the mitochondrion matrix. Since this accumulation is driven by the ΔΨm, a decrease of $DiOC_6$ indicates a loss of this potential. Briefly, 10^6 PMNs were incubated at 39°C for 3 h in the presence of DON and 10 µg/mL LPS in 24-well plates. Then, 50 nM $DiOC_6$ (Molecular Probes, Madison, WI, USA) was added to the wells and the PMNs were incubated for a further 20 min. Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) 40 µM (Enzo Life Sciences, Ann Arbor, MI, USA) was used as positive control. The cells were harvested by centrifugation at 500 x g for 6 min and then suspended in 0.3 mL PBS containing 5 mg/mL BSA and 1 mg/mL NaN_3. $DiOC_6$ fluorescence was recorded using a flow cytometer.

2.8 Measurement of H_2O_2

Hydrogen peroxide content was measured by using a cell permeant probe that becomes fluorescent after reacting with H_2O_2. LPS-stimulated PMNs were cultured in 24-well plates (10^6 cells per well) in DMEM medium and were treated with increasing concentrations of DON for 3 h. 10 µM dichlorodihydro-fluorescein diacetate (H_2DCF-DA) (Molecular Probes, Madison, WI, USA) was then added to the wells and the PMNs were incubated for a further 20 min. 100 µM of the pro-oxidant menadione was used as positive controls. Cells were harvested by centrifugation and suspended in 0.3 mL PBS containing 5 mg/mL BSA and 1 mg/mL NaN_3. Fluorescence was recorded using a flow cytometer.

2.9 Cell protein extraction, SDS-PAGE, and immunoblotting for MAPK and caspase-3 detection

Presence of phosphorylated MAPK and caspase-3 were revealed using Western blot analysis.PMNs (10^6 cells) were treated with increasing concentrations of DON for 30 min (MAPK) or 3 hours (caspase-3) in the presence of 10 µg/mL LPS. Cells were then washed with ice-cold PBS supplemented with a mixture of antiproteases (Roche Diagnostic Corporation, Indianapolis, IN, USA), lysed in 250 µL buffer (2% (w/v) SDS, 1.0 mM sodium ortho-vanadate, DTT, 50mM, 20% glycerol, 0.2% bromophenol blue and 62.5 mM Tris, pH 6.8), and sonicated for 30 seconds. Proteins (10µg per lane) were separated on 12% SDS-PAGE and then transferred to nitrocellulose membrane as already described [24]. The membranes were blocked for 2 hours with PBS-Tween 5% dried skimmed milk and incubated with rabbit anti-human phosphorylated p38, phosphorylated ERK1/2, cleaved caspase-3 or rabbit anti-human β-actin antibodies (Cell Signaling Technology, Danvers, MA, USA) diluted 1:500 in PBS-Tween-milk. After washing, they were incubated with 1:10000 CF^{TM}680 or CF^{TM}770 conjugated anti-rabbit IgG (Biotium, Hayward, CA, USA). Fluorescence intensities were analyzed using an Odyssey Infrared Imaging System (LI-COR; ScienceTec, Les Ulis, France). MAPK and caspase-3 expression was estimated after normalization with the amount of β-actin as already described [25].

2.10 Flow cytometry

All flow cytometry analyses were performed with a MACSQuant Analyser 10 (Miltenyi Biotec, Bergisch Gladbach, D) and the data were calculated using Venturi software (Applied Cytometry, Sheffield, UK).

2.11 Statistical analyses

Data are expressed as mean ± SEM. A significant difference between two data sets was determined using the Holm-Sidak pairwise comparison test (One Way ANOVA test) or paired t-test. Data were calculated using SigmaPlot software (Systat Software, Chicago, IL, USA).

3 Results

3.1 Effect of DON on IL-8 production by PMNs

We first investigated the ability of DON to modulate the synthesis of IL-8. PMNs were stimulated or not with LPS in the presence of increasing concentrations of DON (0.5 to 50 µM) for 3 to 24 hours. After exposure to DON, the cell supernatant was collected and IL-8 concentration measured by ELISA.

LPS is the major molecule linked to the pathogenicity of Gram-negative bacteria, and so incubation with LPS was used to mimic the activation of PMNs during infection. As expected, LPS stimulated the production of IL-8 in non-treated cells (Fig. 1). This stimulation was observed in the supernatants collected after 8 or 24 hours incubation but not at the earlier time point of 3 hours. DON used alone was not able to stimulate IL-8 synthesis (data not shown). When LPS-stimulated PMNs were incubated with increasing concentrations of DON, a dose dependant decrease in IL-8 production was observed. At 0.5 µM and 5 µM, DON decreased the IL-8 concentration in the supernatant by 23% and 93 % respectively when compared to the non-treated PMNs.

3.2 Effect of DON on PMN chemoattraction

We next determined the influence of DON on cell migration. PMNs were incubated with increasing concentrations of DON in the inserts of a transwell culture plates. The number of cells migrating to the lower compartment containing IL-8 as chemoattractant was assessed by measuring the MPO activity (Fig. 2). In the absence of IL-8, the number of migrating cells

was negligible (< 4 x 10^3 cells) and, as expected, IL-8 induced a strong migration of PMNs across the transwell filters (1.05 ± 0.11 x 10^5 cells). DON treatment reduced the migration of PMNs in a dose dependent manner: a significant decrease was observed from 10µM DON (0.82 ± 0.11 x 10^5 cells, p = 0.0021). Treatment with 50µM DON reduced the number of migrated PMNs by 30% when compared to untreated cells.

3.3 Effect of DON on PMN phagocytosis

The effect of DON was investigated on a third immune function of PMNs, their ability to phagocytose microorganism. Cells were treated for 3 hours in the presence of LPS with increasing concentrations of DON and then incubated at 39°C with BODIPY-labelled *Escherichia coli* for 45 min. Phagocytosed bacteria were assessed by flow cytometry. As shown in Figure 3A, when PMNs were incubated on ice, the cells were non-phagocytic and thus a low level of fluorescence was observed. By contrast, when PMNs were incubated at 39°C, the increased mean fluorescence intensity (MFI) demonstrated a phagocytic activity. Exposure to DON led to a loss of fluorescence. As shown in figure 3B, the incubation of PMNs with increasing concentrations of DON (1 to 50 µM) resulted in a strongly dose dependant reduction in their ability to phagocytose labelled *E. coli* (100% inhibition at 50 µM).

3.4 Induction of cell death in pig PMNs after DON exposure

The molecular basis for the impairment of the immune function of PMNs was next determined by examining the mechanism of cell death. We first assessed the lytic effect of DON by measuring the LDH released, but after exposure to 0 – 50 µM mycotoxin, there was no significant effect of the treatment (data not shown). However, an effect of DON on apoptosis was observed. Apoptotic PMNs were detected by flow cytometry, measuring the binding of annexinV to phosphatidylserine externalized to the outer leaflet of the cell membrane. Propidium iodide was used to identify necrotic cells.

As shown in Figure 4A, after 3 hours in the absence of LPS and DON, spontaneous apoptosis was observed in 20.5 ± 4.0 % of the cell population. This proportion remained similar when PMNs were stimulated with 10 µg LPS/mL (18.3 ± 3.4 %). In these LPS treated-PMNs, DON induced early apoptosis (AnnexinV +/ PI-) in a dose dependant manner, with 50 µM resulting in 41.2 ± 4.5 % of apoptotic cells.

Apoptosis was further investigated at a longer time exposure to DON (8 hours). Spontaneous apoptosis, observed without DON treatment was higher in the absence of the survival factor LPS than in its presence. Of note, the high variability observed in non-LPS treated cells might reflect the heterogeneity of the activation state of the isolated PMNs population. As shown in Figure 4B, apoptosis was much more pronounced after 8 hours than after 3 hours exposure to DON. DON increased the number of apoptotic PMNs from 32.8 ± 7.2 % in its absence to 96.0 ± 2.5 % at 50 µM DON. At this concentration, late apoptosis (AnnexinV+/ PI+) could be observed in 30% of the LPS-stimulated PMNs.

The induction of apoptosis by DON was confirmed by the measurement of the mitochondrial transmembrane potential ($\Delta\Psi m$). Indeed, during apoptosis, a reduction in the $\Delta\Psi m$ is an early event caused by the leakage of protons from the intermembrane space of the mitochondria due to the formation of pores in the mitochondrial membrane. Figure 5 shows that 3 hours exposure to DON led to a significant loss of mitochondrial potential in PMNs from 5 µM DON. However, the loss of mitochondrial potential was not strongly enhanced at a ten-fold higher concentration of DON.

Activation of the effector caspase-3 is one of the key events that occur after the mitochondrial membrane permeability. Indeed, release of cytochrome C from the inner mitochondrial membrane contributes to the formation of the apoptosome and the subsequent activation of

the caspase cascade. The activated caspase-3 was measured by Western blot after 3 hours exposure to DON. The level of activated caspase-3 increased in PMNs exposed to DON in a dose dependant manner (Fig. 6) confirming the induction of the apoptotic pathway from 5 µM exposure to the toxin.

3.5 Effect of DON on the hydrogen peroxide content

Reactive oxygen species (ROS), like hydrogen peroxide (H_2O_2), play a role in the destructive process of phagocytosed microorganisms and ROS are also known to be a signal of the onset of apoptosis. Therefore we used fluorescent probes to measure the formation of H_2O_2 following 3 hours exposure to increasing concentrations of DON. Figure 7 shows that LPS induced H_2O_2 production but the addition of DON did not trigger either an inhibition or an increased production of H_2O_2 at concentrations that induced apoptosis. These data indicates that H_2O_2 is not involved in DON-induced apoptosis.

3.6 Effect of DON on p38 and ERK1/2 MAPK activation

The involvement of two MAPKs in the upstream signals controlling the apoptosis was investigated in PMNs. Phosphorylated p38 and ERK1/2 were assessed using Western blot analysis after 30 min exposure of PMNs to 5 and 50 µM DON in the presence of LPS. The level of phosphorylated p38 was statistically higher ($p = 0.033$) in unstimulated PMNs when compared to LPS-stimulated PMNs (Fig. 8). DON exposure increased the amount of phosphorylated p38 in LPS-stimulated PMNs beyond the level observed with unstimulated cells ($p = 0.037$ for DON 5µM and $p = 0.030$ for DON 50µM). These results showed that p38 was involved in the initiation of the apoptotic process induced by DON. By contrast, phosphorylation of ERK1/2 was neither affected by the presence of LPS nor by the exposure to DON suggesting that this MAPK was not involved in the apoptotic pathway in pig PMNs.

4 Discussion

Neutrophils are the most abundant type of circulating leukocytes and play a key role in the front-line defence against invading pathogens. They are also committed in the transition between innate and acquired immune responses. In the present study, LPS from *E. coli* was used to mimic bacterial infection and to induce an inflammatory stimulus. LPS is a major structural component of the outer membrane of gram-negative bacteria and a potent immune activator closely associated with many infectious and inflammatory diseases [26]. LPS binding to the PMN membrane receptor CD14 is one of the first steps in the innate immune response [27].

The data presented herein demonstrated that DON impairs three main functions of porcine PMNs: their ability to migrate to an inflamed site, their phagocytic capacity and their production of IL-8. This alteration of the PMNs immune functions was concomitant with the induction of apoptosis.

PMNs are able to migrate toward sites of inflammation following chemotactic gradients. In the present work, we demonstrated that *in vitro* exposure of porcine PMNs to 10-50 µM DON decreased their chemotaxis towards IL-8. This may lead to an impaired mobilization and recruitment of these leukocytes during an infection and thus increase the susceptibility to infection [28]. Very few studies have investigated the effect of mycotoxins on PMN chemotaxis. Using formyl-Met-Leu-Phe as chemoattractant, an inhibitory effect of ochratoxin B and zearalenone was observed at low concentration (3 nM to 30 µM) on the chemotaxis of human neutrophils [17]. A previous study showed that the random migration of porcine neutrophils was not affected by low concentrations (0.1 to 10 µM) of DON [18] suggesting that DON interfered with the signal between chemoattractant and the PMNs but did not affect the capacity of PMNs to move in the surroundings.

PMNs are recruited to sites of infection in order to ingest microorganisms and to destroy them through the action of ROS and the antimicrobial content of the phagocytic granules. We showed that DON considerably reduced the phagocytic capacity of PMNs but did not alter the H_2O_2 content of the cells. Additionally, cellular levels of nitric oxide were not affected by DON exposure (data not shown). Our results are consistent with previous studies showing that DON decreased the phagocytic activity of macrophages isolated from mice [29] or porcine monocyte-derived dendritic cells [16].

Activation of neutrophils by inflammatory stimuli such as LPS induces the release of a variety of proteins, including cytokines that are involved in PMN effector functions [19]. Among these cytokines, IL-8 is the cytokine most abundantly secreted by neutrophils, and on the other hand, it is a potent chemoattractant for PMNs [28]. Numerous studies have demonstrated that DON and other trichothecenes can cause either immune stimulation or suppression in leukocytes depending on the dose, frequency and duration of exposure [30]. In the current study, IL-8 secretion of PMNs in response to LPS stimulation is consistent with studies realized on porcine [31] or human PMNs [32]. We demonstrated that DON inhibited IL-8 secretion by LPS-stimulated PMNs from low concentration (0.5 µM) and that this inhibition was total at 5 µM. On the contrary, in a human clonal macrophage model, 3.2 µM DON was shown to increase the secretion of IL-8 from 6 to 48h in a synergetic manner when co-incubated with LPS [33]. However, the same concentration of DON suppressed the LPS induced IL-6 production. Such synergistic and inhibiting effects of co-exposure to DON and LPS regarding the secretion of TNF-alpha and IL-6 were observed in porcine pulmonary alveolar macrophages [34]. By contrast, others studies showed that low concentrations of DON ranging from 0.01µM to 1µM decreased the LPS-induced production of IL-10 and IL-12 by human [35], and porcine dendritic cells [16]. Thus, the effect of DON on the LPS-induced IL-8 production by PMNs could not be predicted from published papers since the effect of DON on cytokine synthesis differs with the cell type and the cytokine studied.

Tricothecenes are known to induce apoptosis in various cells, notably in leukocytes such as macrophages [36], monocytes [37], B-cells**,** T-cells, IgA+ cells [38], and erythroleukemia cell line K562 [39]. The current study demonstrates that DON also induces apoptosis in PMNs. We further investigated the mechanism of action of this toxin and observed that DON induced apoptosis via a process involving the permeabilization of the mitochondrial membrane, the activation of caspase-3 and the translocation of phosphatidylserine to the outer leaflet of the cell membrane. This latter event is known to enable macrophages to recognize apoptotic cells by binding to their phosphatidylserine receptors [40].

ROS have a dual function in neutrophils: they play a destructive role in response to inflammation but they may also be involved in the apoptotic process through activation of caspase-8 [41]. Our study shows that the stimulation of PMNs with LPS induced the production of H_2O_2 but it was not modified by DON exposure. Since DON did not interfere with the cellular level of H_2O_2, ROS should not be involved in the apoptotic process. These data are in accordance with the results obtained by Bensassi et al. on intestinal epithelial cells [42]. In this latter study, 10 µM DON induced apoptosis but did not affect H_2O_2 cell content. Data obtained in macrophages also suggest that H_2O_2 is not involved in apoptosis induced by DON. Indeed, when used at 0.85 µM, DON triggered apoptosis while lowering the cell content of H_2O_2 [43].

It was previously demonstrated that the cytotoxic and apoptotic capacities of trichotecenes were related to activation of MAPKs in murine macrophages, human leukemic cells [44] and porcine intestinal epithelial cells [25]. The three major types of MAPK have been detected in human neutrophils (ERK, p38, JNK) but only ERK and p38 have been shown to be involved in neutrophil apoptosis [45]. Our results show that in pig PMNs, in the first minutes of exposure DON induced phosphorylation of p38 but not ERK1/2. Furthermore, these events

are correlated with the induction of apoptosis within 3 hours and an inhibition of IL-8 secretion later on. To confirm the possible key role of p38 in the toxic process induced by DON, it should be interesting to check that the presence of a p38 inhibitor could counteract the activation of apoptosis. With the exception of ERK1/2 phosphorylation, our results are in accordance with the previous studies on murine macrophage [36, 46] using a low dose of DON (0.85 μM). These latter studies demonstrated that DON induced a cascade of events leading to apoptosis: p38 and ERK phosphorylation, p53 phosphorylation, and mitochondria permeabilization, resulting in the release of cytochrome C, and caspase-3 activation, with a peak of activity appearing after 4 hours of exposure.

In conclusion, PMNs play a major role in innate defences and mediate a rapid response against invading microorganisms. Our data showed that exposure to DON resulted in the alteration of three main functions of PMNs: secretion of inflammatory mediators such as IL-8, chemotaxis and phagocytic ability. This impairment of the immune functions of PMNs is correlated with an apoptotic process that is mediated by p38 MAPK. Thus the toxic effect of DON on PMNs should slow down the elimination of pathogens and consequently increase the susceptibility of the host to infection.

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Figures

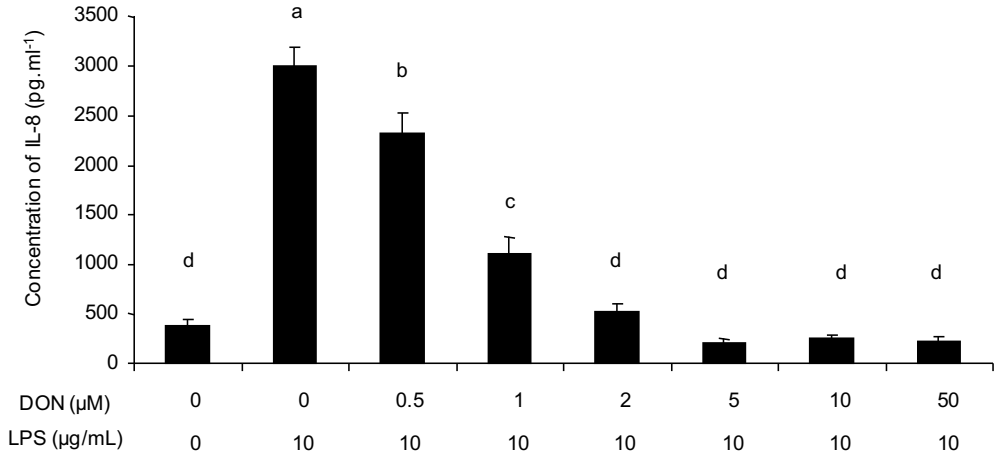

Figure 1: Effect of DON on the production of IL-8 by porcine PMNs. PMNs stimulated with 10 µg/mL LPS were incubated with increasing concentrations of DON for 24 hours. Culture supernatants were collected and the concentration of IL-8 was measured by ELISA. Results from 6 independent experiments are expressed as mean ± SEM. Data not sharing the same letter are significantly different ($p < 0.05$).

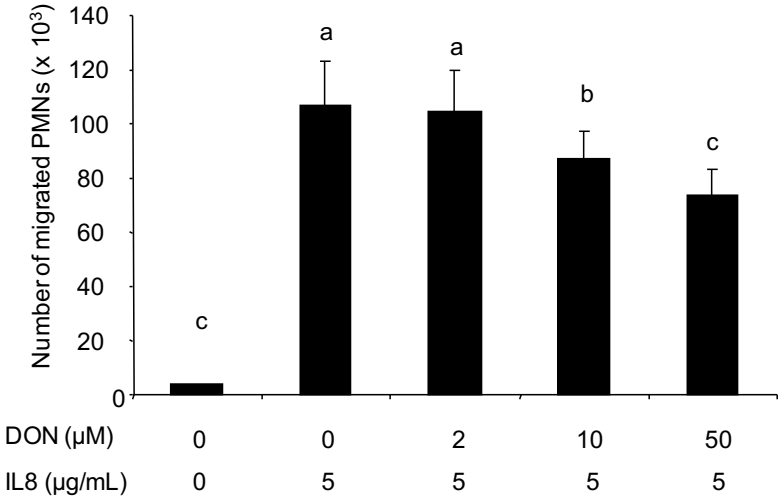

Figure 2: Effect of DON on the migration of PMNs. PMNs were incubated for 1 hour with 0 to 50µM DON and then transferred to the upper compartment of transwell filters containing IL-8 as a chemoattractant in the lower compartment. 2 hours later the number of migrating PMNs was assessed using the MPO assay. Data are expressed as number of migrated PMNs (mean ± SEM, n=6). Data not sharing the same letter are significantly different ($p < 0.05$).

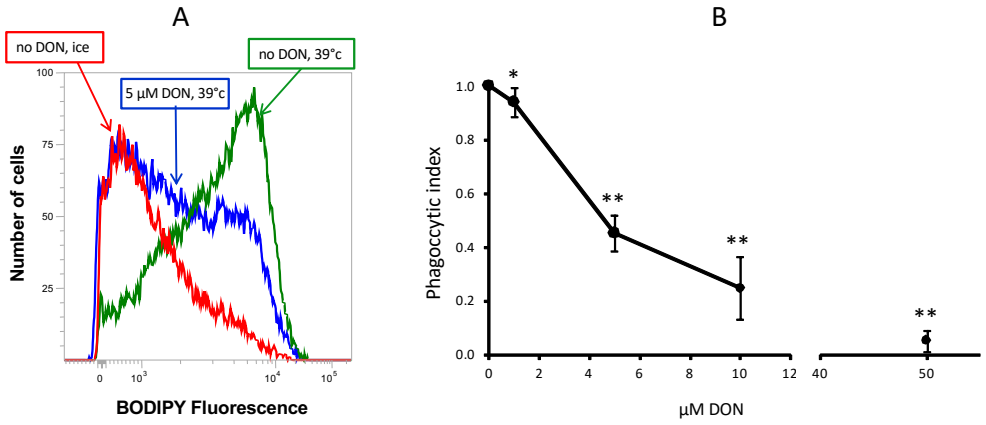

Figure 3: Effect of DON on the phagocytic activity of PMNs. PMNs were exposed to LPS and increasing concentrations of DON for 3 hours. After incubation with BODIPY-labeled *Escherichia coli* the phagocytosis was analyzed by flow cytometry. Figure A: representative histogram of BODIPY fluorescence. Figure B: Phagocytic index from 4 independent experiments. * (p = 0.015) and ** (p < 0.001) mean statistically different from not treated cells.

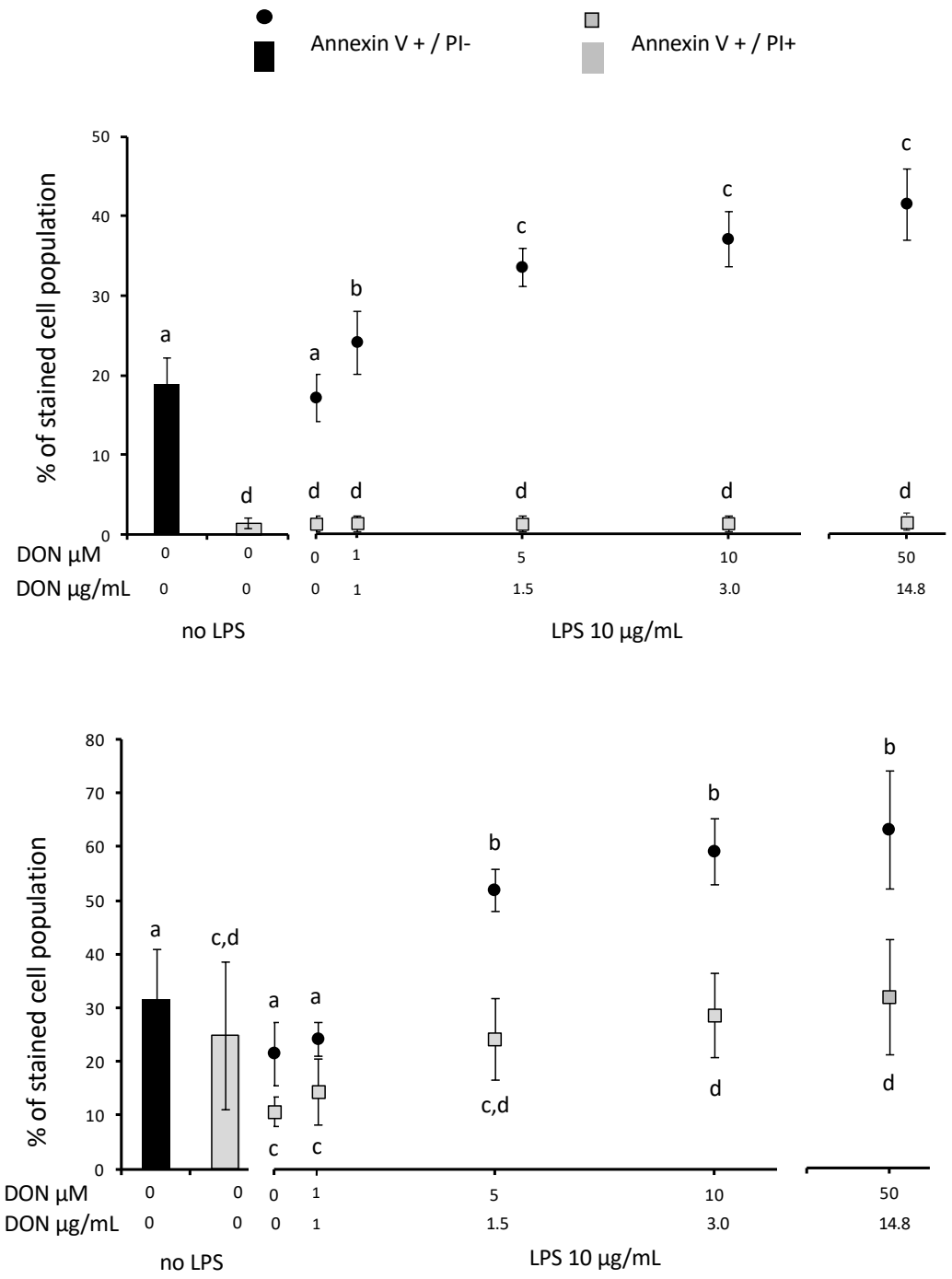

Figure 4: Apoptosis induced in PMNs after 3 hours (A) and 8 hours (B) exposure to DON. PMNs stimulated with LPS and exposed to 0-50 µM DON were stained with AnnexinV-Alexa488 and PI. Results are expressed as percentage of cells from 3-5 independent experiments. Data not sharing the same letter are significantly different ($p < 0.05$).

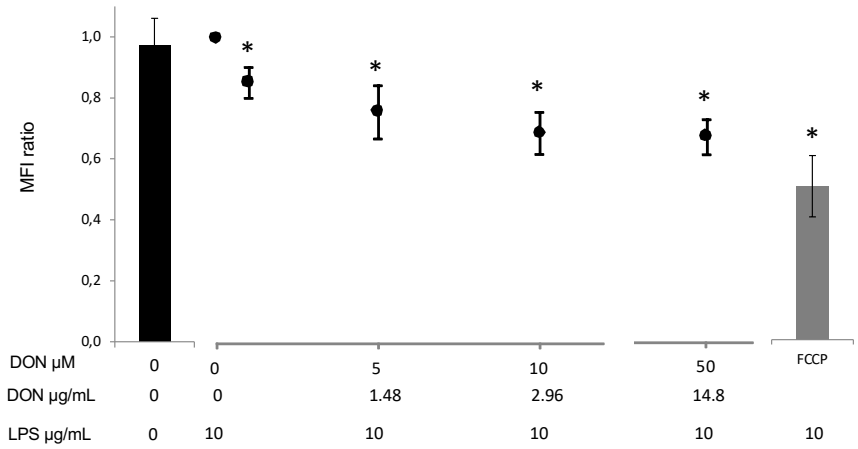

Figure 5: Effect of DON on the mitochondrial transmembrane potential (ΔΨm) in PMNs. LPS stimulated PMNs were exposed to 0- 50 µM DON for 3 hours. After incubation with $DioC_6$ the ΔΨm was recorded by flow cytometry. Results from 3 experiments are expressed as the ratio of MFI in the presence and in the absence of DON. * means statistically different from not treated cells ($p < 0.001$).

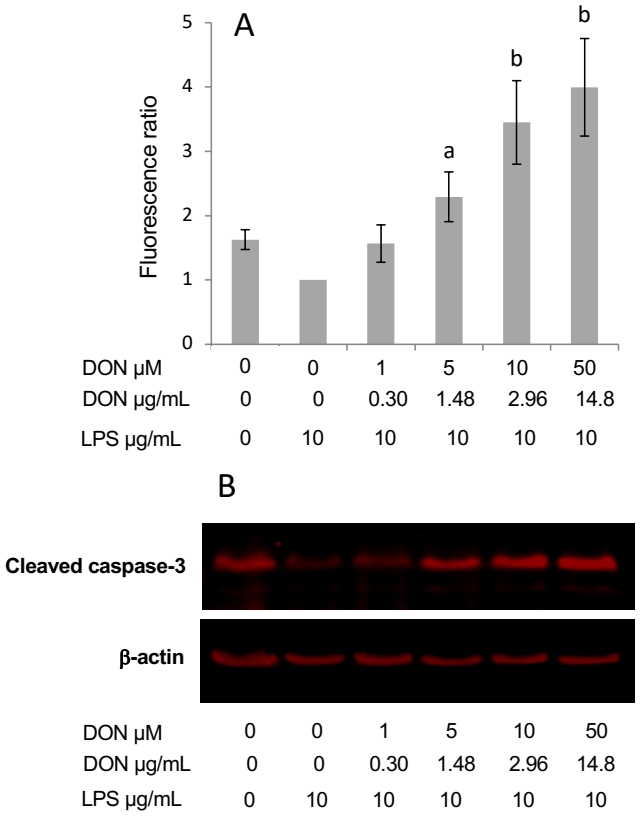

Figure 6: Effect of DON on the activation of caspase-3. PMNs were exposed to increasing concentrations of DON for 3 hours. Cell lysates were analysed by Western blot for the presence of cleaved caspase-3 and β-actin. Panel A represents the results obtained when dividing each data with that obtained with the control (10 µg/mL LPS, no DON). a and b mean significantly different (n=4, $p = 0.005$ and $p < 0.001$ respectively) from the control. Panel B shows representative Western blots.

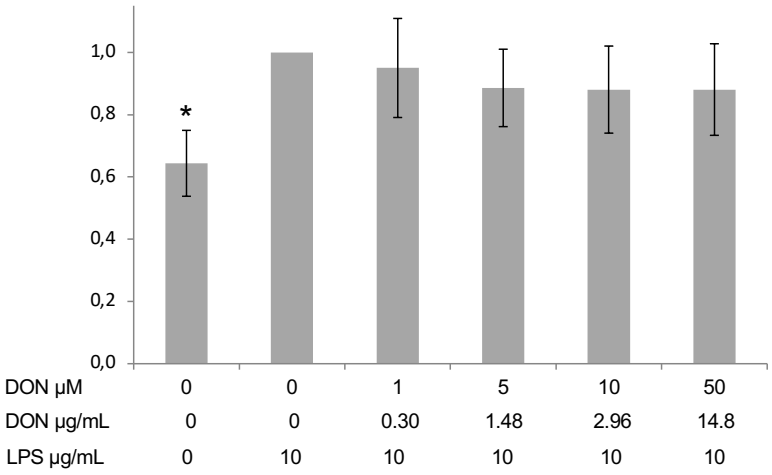

Figure 7: Effect of DON on the level of H_2O_2 in porcine PMNs. PMNs were treated with 0-50 µM DON for 3 hours in the presence of LPS, and further exposed to H_2DCF-DA. H_2O_2 was measured by flow cytometry. Results from 5 independent experiments are expressed as the resulting ratio when dividing each MFI with the MFI obtained with LPS-stimulated controls (n=5, * $p < 0.001$).

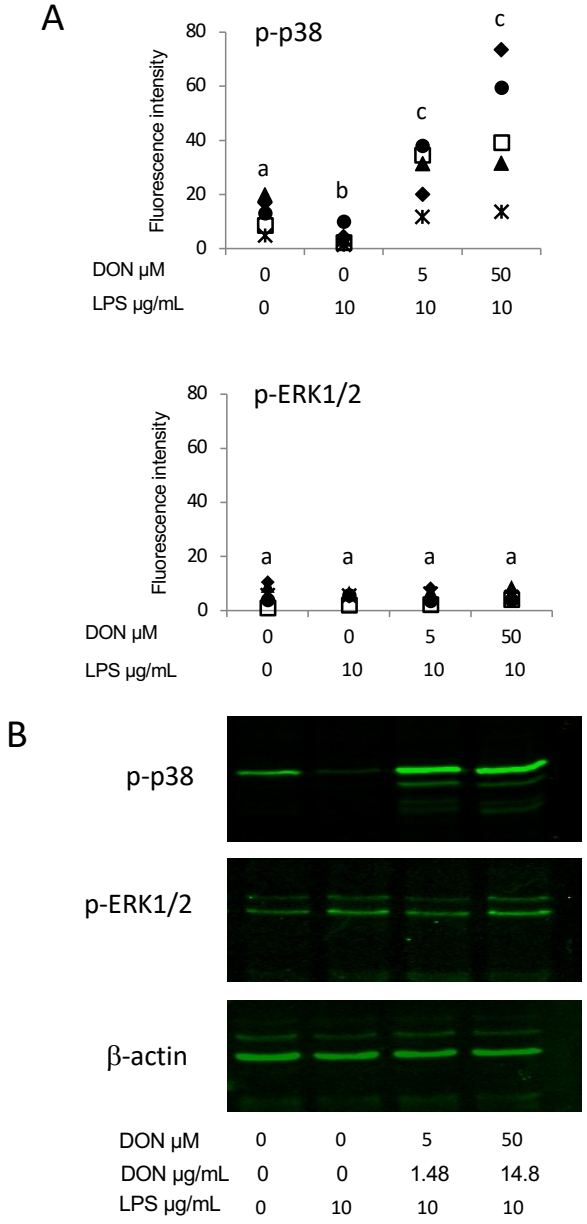

Figure 8: MAPK phosphorylation induced by DON exposure. PMNs were exposed to 0-50 µM DON for 30 minutes in the presence LPS. Cell lysates were analysed by Western blot using antibodies specific for phosphorylated p38 and phosphylated ERK1/2. Results in A are expressed as a ratio between MAPK and β-actin. Each symbol represents results of an independent experiment. Data not sharing the same letter are significantly different (pairwise ANOVA test, n=5, $p < 0.05$). Panel B shows representative Western blots.