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Trichothecenes NIV and DON modulate the maturation of murine dendritic cells

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ABSTRACT

Nivalenol (NIV) and Deoxynivalenol (DON), mycotoxins of the trichothecene family are considered very common food contaminants. In this work, we investigated whether the immunotoxic effects ascribed to these trichothecenes may be mediated by perturbations in the activity of dendritic cells (DCs). Murine bone marrow-derived DCs were used to evaluate the effects of NIV and DON on the LPS-induced maturation process. We found that the expression of the class II MHC and of the accessory CD11c molecules, but not of the costimulatory CD86 marker, was down-regulated by NIV and DON exposure in LPS-treated DCs, as well as nitric oxide (NO) production. Interestingly, NIV, but not DON, induced DC necrosis. Moreover, the analysis of the cytokine pattern showed that IL-12 and IL-10 expressions induced by LPS exposure were suppressed by both trichothecenes in a dose-dependent fashion. On the other hand, the secretion of the proinflammatory cytokine TNF-α was increased as a direct consequence of DON and NIV exposure. Taken together, our data indicated that the immunotoxicity of NIV and DON was related to the capacity of both trichothecenes to interfere with phenotypic and functional features of maturing DCs.

1. Introduction

Mycotoxin contamination of foods and feeds gained much global attention in recent years owing to its adverse health and economic effects. Nivalenol (NIV) and Deoxynivalenol (DON) are mycotoxins of trichothecene family produced by *Fusarium* fungi, such as *Fusarium graminearum*, commonly present in foods and feeds of cereal origin (Rotter et al., 1996; Placinta et al., 1999). A recent data collection on the occurrence of *Fusarium* toxins in food in the European Union showed a 57% incidence of positive samples for DON and 16% for NIV out of several thousands of samples analyzed (Schothorst and van Egmond, 2004). DON and NIV contaminations are reported to contribute to gastrointestinal diseases in exposed humans (Li et al., 1999). Trichothecenes cause several toxic effects including anorexia, vomiting, fever and nausea but also affect the immune system. DON consumption causes acute and chronic toxicity and influence animal feeding behavior and immunity (Lautraite et al., 1997; Schlatter, 2004; Pestka et al., 2004). Literature data attribute to trichothecenes both stimulating and suppressing activities on the immune function, depending upon the dose and duration of exposure (Bondy and Pestka, 2000). Low dose exposure generally results in stimulatory effects causing an increased resistance to pathogens, elevated serum IgA levels and up-regulated expression of genes encoding for cytokines and chemokines. On the contrary, high dose exposure causes immunosuppression, characterized by decreased resistance to

Abbreviations: BM, bone marrow; DCs, dendritic cells; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte–macrophage colony-stimulating factor; LPS, lipopolysaccharide; PE, phycoerythrin; PBS, phosphate buffered saline.

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pathogens, reduced IgM and IgG levels and impaired delayed type hypersensitivity responses. In particular, studies on macrophages, T and B cells, showed that the trichothecene-mediated immunosuppressive effect was associated to induction of apoptosis (Pestka et al., 1994; Shifrin and Anderson, 1999; Rocha et al., 2005). To date a very limited number of studies on NIV immunotoxicity have been carried out. NIV was found to inhibit proliferation of human mitogen-stimulated lymphocytes (Thuvander et al., 1999; Forsell and Pestka, 1985). In addition, *in vivo* studies showed that NIV suppressed both total and antigen-specific IgE production in TcR transgenic mice (Choi et al., 2000).

Dendritic cells (DCs) are considered the most potent antigen presenting cells (APCs) and are critically involved in initiating adaptive immune responses. DCs are present in many organs, such as skin, intestine and respiratory tract, in a immature state characterized by high level of endocytosis activity and reduced expression of adhesion, costimulatory and major histocompatibility complex (MHC) class II molecules. The role of adhesion molecules is to favor a prolonged contact between DCs and T cells, whereas costimulatory molecules signal T cells to proliferate and differentiate (Inaba et al., 1994). Following antigen capture, DCs start the maturation process characterized by migration of DCs toward T-cell areas of secondary lymphoid organs and by acquisition of the capacity to present antigens to naïve T cells. In this mature state, DCs down-regulate their phagocytic activity and increase the expression of MHC (classes I and II), adhesion and costimulatory molecules (CD40, CD80, CD86). All these surface markers act in concert and provide the essential secondary signals for initiating immune responses. The maturation process comprises also crucial functional changes, such as induction of cytokine production (IL-12, IL-10, TNF-α) and of oxygen reactive species as nitric oxide (NO), that are able to influence the phenotype of the adaptive response. Interestingly, DC maturation can be induced *in vitro* by various stimuli, such as proinflammatory cytokines (TNF-α and IL-1β) and bacterial products (LPS) (Versasselt et al., 1997), so providing a model to analyze substances interfering with this pivotal event in the control of adaptive immunity.

The objective of present study was to establish whether *in vitro* exposure of murine bone marrow-derived DCs to NIV and DON can interfere with the maturation process. Our results showed that both toxins were capable to influence the expression of maturation markers, NO production, cell viability and cytokine secretion. These effects could explain the known immunomodulatory activity of trichothecenes.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium, L-glutamine, streptomycin, penicillin, fetal bovine serum (FBS), phosphate buffered saline (PBS) and non-essential amino acids were from Cambrex Bioproducts Europe (Verviers, Belgium). Caspase-3 substrate, acetyl Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), and caspase-3 standard, 7-amino-4-methylcoumarin (AMC), were obtained from Calbiochem (San Diego, CA, USA). Granulocyte–macrophage colony-stimulating factor (GM-CSF), DON (D-0156), NIV (N-7769), β-mercaptoethanol, lipopolysaccharide (LPS), reagents for LDH assay, antibiotics and all other chemicals were purchased from SIGMA (St. Louis, MO, USA).

2.2. Isolation of dendritic cells

BALB/c mice (Charles River, Calco Lecco, Italy) were maintained in pathogen-free conditions at our animal facility. DCs were prepared according to Lutz et al. (1999). In brief, femurs and tibiae of 8–12 weeks old mice were removed from the surrounding muscle tissue, left in 70% ethanol for 2–5 min and washed with PBS. Then, both bone ends were cut and the bone marrow (BM) flushed with PBS. BM leukocytes were seeded at 6×10^5/well in 6-well plates, in 2 ml RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μM β-mercaptoethanol and 10% heat-inactivated fetal calf serum (FCS). 20 ng/ml (200 U/ml) GM-CSF was added as specific growth factor. On day 3, a further 2 ml of medium containing 200 U/ml GM-CSF were added to the wells. On day 7, half of the supernatant was carefully collected, centrifuged and cell pellet re-suspended in 2 ml fresh medium enriched with GM-CSF (200 U/ml) and given back into wells. On day 10, cells were treated as on day 7, but only 50 U/ml GM-CSF was added to fresh medium. At this stage cells were incubated with mycotoxins or LPS.

2.3. Exposure to trichothecenes

DON and NIV were dissolved in ethanol and methanol, respectively to make 10 mM stock solutions. Work solutions were made by direct dilution in culture medium. Immature DCs were exposed for 6 h to increasing concentrations of each mycotoxin before adding LPS (1 μg/ml) for further 18 h. In each experiment, untreated cells and cells incubated with LPS alone were used as negative and positive controls, respectively. Cell number and viability were assessed by nigrosin dye exclusion assay using a hemocytometer.

2.4. Apoptosis assay

The release of 7-amino-4-methylcoumarin (AMC), following enzyme hydrolysis of acetyl Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was used to determine the caspase-3 activity. After the different treatments, cells were washed with PBS and resuspended in 100 μl of 50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT (lysis buffer) for 20 min at 4 °C. The obtained lysates were centrifuged (10,000 × *g*, 5 min, 4 °C) and supernatant aliquots (15 μg) were incubated (60 min at 37 °C) with reaction buffer (lysis buffer supplemented with 2 mM EDTA and 50 μM Ac-DEVD-AMC). At the end of this period, fluorescence ($360_{Ex}/460_{EM}$) was recorded in both samples and quantitated by means of a standard curve prepared

with pure AMC. Caspase-3 activity was finally expressed as nmoles of AMC/mg protein/h. Cells treated with 10 μM actinomycin D were used as positive control.

2.5. Cytotoxicity assay

Lactate dehydrogenase (LDH) was used as marker for cellular toxicity (Tipton et al., 2003). Cell suspensions were centrifuged (4000 × *g*, 5 min, 4 °C) and the supernatants were recovered, whereas the pellets were lysed in 100 μl of 0.2 M Tris/HCl pH 8.0, containing 10% (w/v) Triton X-100, for 30 min at 4 °C. Lysates and supernatant aliquots (100 μl) were then incubated with 100 μl reaction buffer (0.7 mM p-iodonitrotetrazolium violet, 50 mM L-lactic acid, 0.3 mM phenazine methoxysulphate, 0.4 mM NAD, 0.2 M Tris/HCl pH 8.0) for 30 min at 37 °C. Absorbance was read at 490 nm; results were expressed as percentage of total LDH release from control cultures treated with 1% (w/v) Triton X-100 and calculated as follows: [(experimental value − blank value)/(total lysis − blank value) × 100].

2.6. FACS analysis

DCs (about 1×10^5), washed and resuspended in cold buffer (PBS supplemented with 2% FBS) were incubated with different fluorescein isothiocyanate (FITC)- phycoerythrin (PE)-or allophycocyanin (APC)-conjugated monoclonal antibodies (mAb) (BD Pharmingen, San Diego, CA, USA), for 30 min at 4 °C. Appropriately labelled isotype controls were used to determine non-specific fluorescence. Stained cells were then washed twice and resuspended in cold buffer and assayed using a CyFlow Space flow cytometer (Partec, Munster, Germany). At least ten-thousand cells were analyzed for each sample and data were processed by using FlowJo software (Tree Star Inc, Ashland, OR, USA).

2.7. Cytokine secretion

TNF-α, IL-12 p70 and IL-10 concentrations in cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA) essentially according to a published protocol (Mauriello et al., 2007). In brief, 100 μl of capture antibody solution (BD Pharmingen, San Diego, CA, USA) were plated into ELISA wells and incubated overnight at 4 °C. After the removal of the antibody solution, 100 μl aliquots of phosphate buffered saline supplemented with 2% BSA were added and incubated at room temperature (r.t.) for 2 h. Next, cytokine standard and samples, diluted in blocking buffer supplemented with 0.05% Tween-20 were incubated overnight at 4 °C. At the end of the incubation, 100 μl aliquots of biotinylated antibody solution were plated and left for 2 h at r.t. Streptavidin–horseradish peroxidase conjugate IgGs (1:2000 dilution) (BD Pharmingen) was incubated for 1 h at r.t. and, finally, 200 μl aliquots of 63 mM Na_2HPO_4, 29 mM citric acid (pH 6.0) containing 0.66 mg/ml *o*-phenylenediamine/HCl and 0.05% hydrogen peroxide were dispensed into each well and allowed to develop. The absorbance was finally read at 450 nm with a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The cytokine concentrations were calculated by using appropriate standard curves and expressed as pg/ml.

2.8. Nitric oxide (NO) assay

Nitrite accumulation was used as an indication of NO production. Nitrite concentration was determined according to a previous published procedure (Green et al., 1982). In brief, culture supernatants (100 μl) were placed in a 96 wells plate and mixed with an equal volume of Griess reagent [0.1 naphthyl ethylenediamine (w/v) and 1% (w/v) sulphanilamide in 5% phosphoric acid]. The mixture was incubated for 30 min at room temperature and then the absorbance was read at 550 nm with a microplate reader. Values were calculated by using a standard curve and expressed as μM NO.

2.9. Statistical analysis

All data are presented as the mean ± SD. Differences among the various treatment groups were determined by one-way analysis of variance (ANOVA). Significant differences were found by using the Tukey test. The level of $P < 0.05$ was selected to denote a significant difference.

3. Results

3.1. Trichothecenes down-regulate DC maturation

The expression of molecules such as class II MHC and CD86 is well known to be indicative of the maturation stage of DCs. In this frame, the influence of NIV and DON on DC maturation was evaluated on DCs pre-treated (6 h) with increasing concentrations of these two trichothecenes, before incubation with 1 μg/ml LPS for further 18 h. Untreated cultures and those exposed to LPS alone were used as negative and positive controls, respectively. As expected, class II MHC expression was up-regulated by LPS, whereas pre-treatment with a high dose (3 μM) of DON or NIV reduced its expression (Fig. 1A). Lower doses of mycotoxins did not apparently influence MHC expression (data not shown). In accordance with these results, we found that the percentage of CD11c CD86 double positive cells, representing the population of mature DCs, showed a significant decrease in cultures exposed to 3 μM DON or 2 μM NIV (Fig. 1B). Dot plot analysis showed that LPS preferentially induced CD86 expression in double positive cells. On the contrary, we found that the decrease of mature DCs mediated by both toxins was essentially related to a reduced expression of the accessory molecule CD11c (Fig. 1C).

As nitric oxide (NO) production normally occurred in response to LPS-induced maturation (Lu et al., 1996), nitrite production was measured in differently treated DCs to further corroborate the existence of a modulatory effect of trichothecenes on LPS-induced maturation. The significant increase of NO concentration in the culture media of LPS-treated DCs was evidenced and it resulted greatly (two-fold) reduced by pre-incubation with 1 μM DON or NIV (Fig. 2). In addition, NO was almost completely inhibited by the highest concentrations of mycotoxins.

3.2. NIV toxicity reduces DC viability

Next, the influence of NIV and DON on DC viability was examined. DON did not exert any effect on cell viability

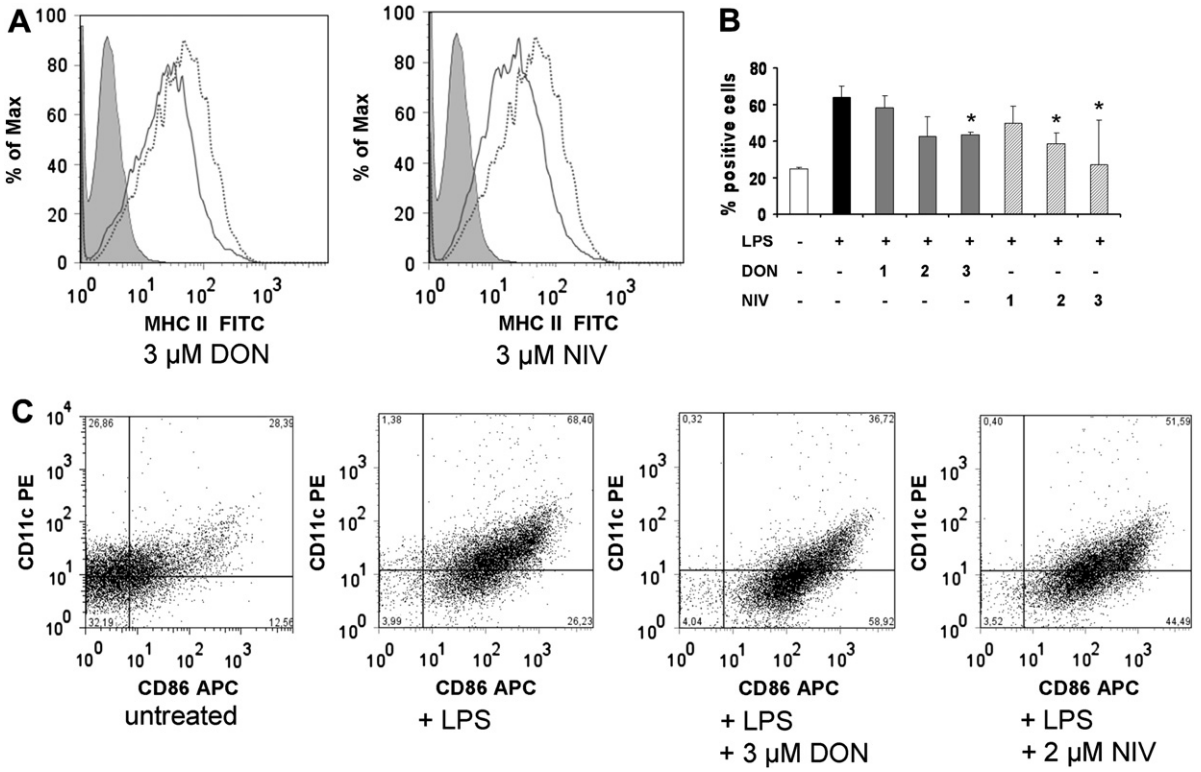

Fig. 1. FACS analysis of maturing DCs (LPS-treated) in the presence of NIV and DON. (A) Class II MHC expression in DCs pre-incubated (6 h) with 3 μM DON or NIV; full gray curve, isotype control; solid line, mycotoxin-pre-treated DCs, dotted line, positive control (LPS-treated alone). (B) Percentage of mature (CD11c CD86) DCs following pre-incubation with increasing concentrations of DON and NIV (1–3 μM); *, different from LPS-treated alone. (C) Dot plot analysis of CD11c and CD86 expression following pre-incubation with 3 μM DON and 2 μM NIV. Results are representative of three experiments.

(Fig. 3A). That was also reflected by no increase in necrosis/apoptosis cell death at any tested dose of DON (Fig. 3B and C). On the contrary, a two- and five-fold decrease of the viable cell number was evidenced in culture exposed to 2 and 3 μM NIV, respectively (Fig. 3A). LDH release was significantly increased by NIV treatment in a dose-dependent fashion (Fig. 3B). Instead, caspase-3 was not activated by NIV (Fig. 3C). Together, these results clearly indicated that cytotoxicity might account for the decreased DC viability mediated by NIV.

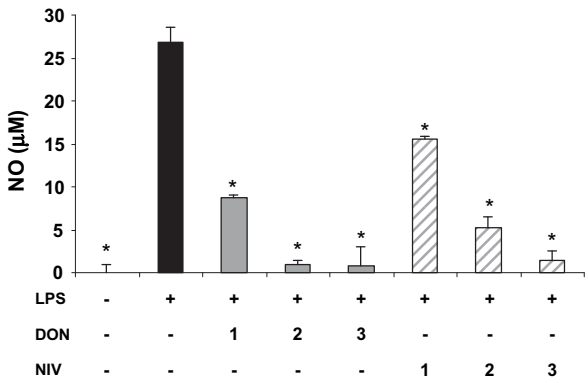

Fig. 2. Effect of trichothecenes on NO production by LPS-stimulated DCs. DCs were pre-treated (6 h) with three different concentrations of mycotoxins (1–3 μM) before addition of LPS (1 μg/ml) for further 18 h. Results were expressed as NO concentration (μM) detected in the culture supernatant. *, Significantly different from positive control (LPS-treated alone). Results are representative of three experiments.

3.3. Trichothecenes modulate cytokine release

As cytokine production by DCs is known to play a key role in the modulation of adaptive response, we measured IL-12, IL-10 and TNF-α secretions operated by DCs under the influence of the two trichothecenes. Presented data evidenced that both IL-10 and IL-12 concentrations were significantly decreased by pre-treatment with 2 and 3 μM DON (Fig. 4), whereas a small but significant increase of TNF-α secretion was measured in culture pre-incubated with 1 and 2 μM DON as compared with the control (Fig. 4). Similarly, a significant reduction of IL-12 and IL-10 release was measured in cultures pre-treated with 2 and 3 μM NIV. Moreover, NIV was shown to elicit the production of significant higher TNF-α amounts in comparison with DON at all the examined concentrations.

4. Discussion

In this work we found that NIV and DON, two mycotoxins commonly detected as food contaminants, were responsible of both phenotypic and functional changes in the maturation process of murine BM-derived dendritic cells (DCs).

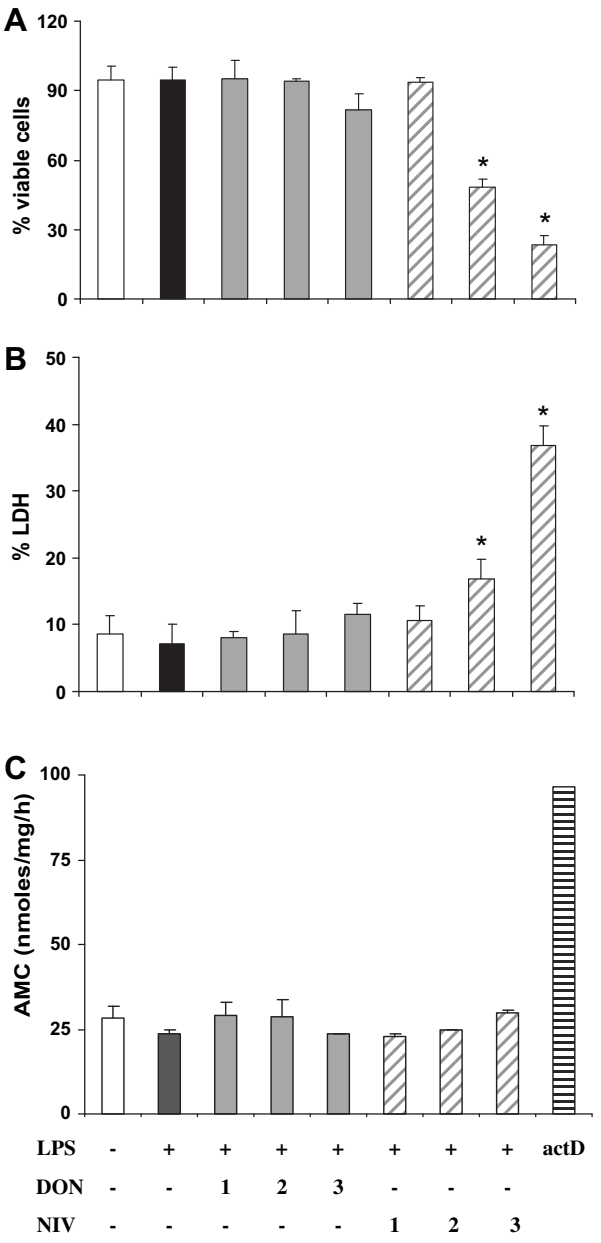

Fig. 3. Effect of trichothecenes on DC viability. DCs were pre-treated (6 h) with three different concentrations of mycotoxins (1–3 μM) before addition of LPS (1 μg/ml) for further 18 h. (A) Cell viability, assessed by nigrosin dye exclusion; (B) cytotoxicity, analyzed by LDH release in culture supernatant; (C) apoptosis, determined by caspase-3 activity and expressed as nmoles of AMC/mg protein/h; ActD, actinomycin D treatment. *, Significantly different from control (LPS-treated alone in A and B, actinomycin-treated in C). Results are representative of three experiments.

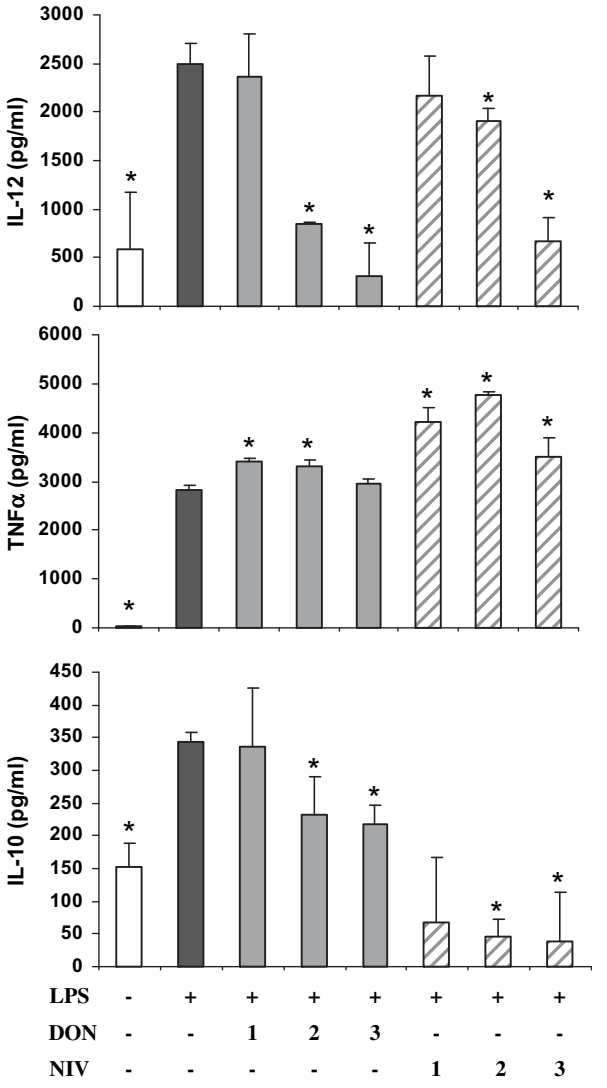

Fig. 4. Cytokine expression of DCs. DCs were pre-treated (6 h) with three different concentrations of mycotoxins (1–3 μM) before addition of LPS (1 μg/ml) for further 18 h. Culture supernatants were collected after 24 h; the levels of IL-12(p70), IL-10 and TNF-α were determined by ELISA and expressed as pg/ml. *, Significantly different from control (LPS-treated alone). Bars represent the mean ± SD of three cultures.

NIV and DON are known to be immunotoxic. DON is the most commonly found trichothecene in human and animal diets, often associated to NIV (Lee et al., 1985; Tanaka et al., 1990). NIV has been reported to induce a higher toxicity than DON on mitogen-induced lymphocyte proliferation (Forsell and Pestka, 1985; Visconti et al., 1991; Severino et al., 2006; Luongo et al., 2008), whereas only a few contrasting data were available on DCs. For this reason we analyzed the effects of both trichothecenes on murine DCs obtained *in vitro* from bone marrow cells. The toxin concentration range was chosen on the basis of previous studies (Sugita-Konishi and Pestka, 2001; Islam and Pestka, 2006; Hymery et al., 2006; Bimczok et al., 2007). Additionally, these values were consistent with the levels that can be found *in vivo* (Yang et al., 2000).

DC maturation is normally induced by antigen engagement or microbial stimuli (e.g. LPS) and it is associated to up-regulation of surface markers. These data were confirmed in our model where LPS induced an increased expression of both class II MHC and CD86 molecules. In this context, our findings highlighted the ability of NIV and DON to influence this maturation step *in vitro*. In particular, both mycotoxins were found to down-regulate class II MHC

surface expression, even if at the highest tested concentration. Similarly, we found a decrease in the number of mature DCs starting at 2 and 3 μM for NIV and DON, respectively. Interestingly, dot plot analysis clearly indicated that this decrease was essentially correlated to a reduced expression of CD11c, but not of the costimulatory molecule CD86, in contrast with previous results on human (Hymery et al., 2006) and porcine (Bimczok et al., 2007) monocyte-derived DCs. CD11c is an accessory molecule whose expression is associated to the development of the mature state (Pinchuk et al., 2007), so suggesting that both mycotoxins targeted a very specific maturation pathway in our model. Then, further experimental data are required to better address this issue.

Nitric oxide (NO) is considered an important effector molecule involved in immune regulation and host defense (Snyder and Bredt, 1992). In particular, DCs (Huang et al., 1999) and monocytes (Perez-Mediavilla et al., 1995) were reported to release NO into the medium. The involvement of NO in the acquisition of a mature phenotype of human DCs has been also demonstrated (Kantengwa et al., 2003). Moreover, NO, along with caspases, has been shown to regulate DC maturation and surface expression of class II MHC proteins (Wong et al., 2004). Altogether, the analysis of these data prompted us to evaluate NO production in DCs pre-treated with different concentrations of the two toxins. In accordance with literature data (Lu et al., 1996), NO production resulted markedly increased in DCs stimulated with LPS. In contrast, pre-exposure of DCs to increasing concentrations of DON and NIV completely abrogated NO production in a dose-dependent way. These data were in accordance with results from Ji et al. (1998), who found DON to be a potent suppressor of LPS-induced NO production in the RAW 264.7 murine cell line. Interestingly, similar effects on LPS-induced NO synthesis were observed in the same macrophage model following treatment with cycloheximide, a known inhibitor of protein synthesis (Weisz et al., 1994). Considering the known capacity of trichothecenes to block protein synthesis through binding to ribosomal peptidyl transferase (Ueno, 1983), our results of inhibition of NO production in murine DCs could be similarly explained.

Interestingly, the assessment of cell viability indicated that, of the two examined toxins, only NIV induced a dose-dependent reduction during the LPS-induced maturation step; LPS by itself did not influence this parameter. In agreement with this result, DON did not induce necrosis, measured as LDH release, or apoptosis (caspase-3 activity) in maturing DCs. That was in contrast with previous *in vitro* and *in vivo* studies where DON-mediated apoptotic effects were documented (Shifrin and Anderson, 1999; Pestka et al., 1994, 2005; Zhou et al., 1999). Such discrepancies could be attributed to the doses of DON we used, lower than in the previously reported works and probably not sufficient to activate caspase-3 or, alternatively, to differences in the examined cell models. Notably, the dose-dependent decrease in cell viability induced by NIV was essentially associated with necrosis, but not with apoptosis induction. This finding could be partially explained by the inhibition of NO in the presence of mycotoxins we found, whose production has been associated to apoptosis (Wong et al., 2004). These data were also in line with the higher expression of surface markers, thus underlying the stronger immunotoxicity of NIV in comparison with DON.

To examine the influence of DON and NIV on functional parameters of DCs, secretion of IL-12, TNF-α and IL-10 was analyzed as representative pro- and anti-inflammatory cytokines. In particular IL-12 is considered a critical Th1-skewing cytokine that elicits IFN-γ production by T cells and by NK cells (Heufler et al., 1996). IL-10 is an anti-inflammatory cytokine that suppresses IL-12 production, thus favoring a Th2/Th3 response and inhibiting T-cell recruitment (Asseman et al., 1999). TNF-α plays a pivotal role in inflammation, mediating fever and inducing the liver to produce various detrimental proteins. In certain immune deficiency disorders, elevated TNF-α production has been demonstrated to be a mediator of pathology. On the other hand, the known cytotoxic action of macrophages was mediated by TNF-α and IL-1 (Rangavajhyala et al., 1997). We found that *in vitro* exposure of DCs to increasing concentrations of DON and NIV significantly abrogated secretion of both IL-12 and IL-10. A similar decrease of cytokine release in response to DON has been previously shown for human DCs (Hymery et al., 2006). NIV apparently inhibited IL-10 secretion to a higher extent than DON, suggesting again its stronger immunotoxicity. Notably, both toxins exacerbated the LPS-induced TNF-α expression, with NIV showing a higher effect, thus driving the overall immune response toward a Th1 phenotype.

In summary, our results highlighted a differential ability of DON and NIV in modulating DC function. Yet, these two compounds have a similar sesquiterpenoid structure, differing only for one more OH-group hold in position 7 by NIV. Literature data encompassed a series of compounds affecting DC functions with effects like those observed in this study. For instance, *in vitro* exposure to polycyclic aromatic hydrocarbons (PAH) exhibited a marked reduction of both phenotypic and functional maturation features (Laupeze et al., 2002). Similar effects were reported in human monocyte-derived DCs with a sequiterpene lactone parthenolide (PTL), known for its anti-inflammatory properties (Uchi et al., 2002). To date, trichothecene effects are preferentially ascribed to their capacity to inhibit protein synthesis. Nevertheless, the exact molecular mechanisms are still unclear at present and will be the subject of future investigations.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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