

## INNATE IMMUNE SENSING OF *Fusarium culmorum* BY MOUSE DENDRITIC CELLS

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Chronic inhalation of grain dust is associated with asthma and chronic bronchitis in grain worker populations. Exposure to fungal particles was postulated to be an important etiologic agent of these pathologies. *Fusarium* species frequently colonize grain and straw and produce a wide array of mycotoxins that impact human health, necessitating an evaluation of risk exposure by inhalation of *Fusarium* and its consequences on immune responses. Data showed that *Fusarium culmorum* is a frequent constituent of aerosols sampled during wheat harvesting in the Vaud region of Switzerland. The aim of this study was to examine cytokine/chemokine responses and innate immune sensing of *F. culmorum* in bone-marrow-derived dendritic cells and macrophages. Overall, dendritic cells and macrophages responded to *F. culmorum* spores but not to its secreted components (i.e., mycotoxins) by releasing large amounts of macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, monocyte chemoattractant protein (MCP)-1, RANTES, and interleukin (IL)-12p40, intermediate amounts of tumor necrosis factor (TNF), IL-6, IL-12p70, IL-33, granulocyte colony-stimulating factor (G-CSF), and interferon gamma-induced protein (IP-10), but no detectable amounts of IL-4 and IL-10, a pattern of mediators compatible with generation of Th1 or Th17 antifungal protective immune responses rather than with Th2-dependent allergic responses. The sensing of *F. culmorum* spores by dendritic cells required dectin-1, the main pattern recognition receptor involved in  $\beta$ -glucans detection, but likely not MyD88 and TRIF-dependent Toll-like receptors. Taken together, our results indicate that *F. culmorum* stimulates potently innate immune cells in a dectin-1-dependent manner, suggesting that inhalation of *F. culmorum* from grain dust may promote immune-related airway diseases in exposed worker populations.

Chronic inhalation of environmental microscopic fungal particles at high concentrations is associated with persistent lung inflammation and respiratory disorders in different occupational populations (Poole et al., 2012; Vacher et al., 2014). In particular, grain workers who are regularly exposed to grain dust (a complex mixture of fungal particles, bacteria, insect compounds, animal wastes, inorganic compounds–silicates, chemicals, gases, and fumes) are known to have reduced lung

volume and display symptoms commonly associated with chronic bronchitis, asthma, and asthma-like disorder (Linaker and Smedley, 2002; Rask-Andersen, 2011; Eduard et al., 2009).

One fungal genus in grain dust is of particular concern for human health: *Fusarium*. This genus includes a number of economically important plant pathogenic species that are responsible for crown rot, root rot, and *Fusarium* head blight (FHB), a

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destructive disease of cereal grain crops worldwide (Bottalico and Perrone, 2002; Lanoue et al., 2010; Stephens et al., 2008). Among the *Fusarium* species, *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* produce a range of mycotoxins that are a serious human health concern when ingested (Bottalico and Perrone, 2002; Foroud and Eudes, 2009; Grenier and Applegate, 2013). The trichothecenes such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), T-2 toxin, and fusarenon-X and the estrogenic metabolite zearalenone (ZEA) are mycotoxins for whose cytotoxicity and immunosuppressive activities are the best described (Bondy and Pestka, 2000; Antonissen et al., 2014). Curiously, although *Fusarium* species are likely to be present in grain dusts, little is known regarding their frequencies in grain aerosols or their capacity to elicit innate immune responses and impact human health upon inhalation.

The development of microbial-induced airway pathologies is largely conditioned by the initial local immune response. Innate immune cells such as alveolar macrophages lining the respiratory tract and dendritic cells (DC) located within the epithelium and interstitium of the lungs form the primary line of defenses against invading microorganisms. These cells sense microorganisms via pattern recognition receptors (PRR) that are specialized in the recognition of so-called microbial associated molecular patterns (MAMP), in the case of fungi principally  $\beta$ -glucans, mannans, and chitin. The most important families of PRR involved in the sensing of fungal MAMP are C-type lectin receptors (CLR, such as dectin-1, dectin-2, DC-SIGN, the mannose receptor, the mannose binding lectin, mincle, and galectin-3), Toll-like receptors (TLR, mainly TLR2, TLR4, and TLR9), and Nod-like receptors (NLR) (Romani, 2011; Brown, 2011). Dectin-1 is the primary receptor involved in sensing of  $\beta$ -glucans, whereas TLR2 senses phospholipomannans, TLR4 O-branched mannans and glucuronoxylomannan, and TLR9 fungal DNA (Brown and Gordon, 2001; Jouault et al., 2003; Shoham

et al., 2001; Netea et al., 2006). Upon microbial sensing, innate immune cells produce a panel of cytokines, chemokines, and other immunomodulatory molecules that play a key role in initiation, amplification, and regulation of innate and adaptive immune responses. Dendritic cells (DC) orchestrate these events by shaping the polarization of naive CD4<sup>+</sup> T helper (Th) cells into Th1, Th2, or Th17 effector T cells that exert different impacts on antifungal responses. Th1 and Th17 cells promote protective immunity and fungal clearance, while Th2 cells favor development of fungal allergic responses (Romani, 2011).

Although an increasing number of environmental fungal species are associated with allergic and nonallergic respiratory disorders when inhaled, the innate immune responses triggered and mechanisms underlying their recognition by innate immune cells remain poorly investigated. Given this context, the aim of this study was to characterize the response of innate immune cells, in particular DC, to *F. culmorum*, identified as the most common *Fusarium* species present in wheat-associated aerosols from the Vaud area of Switzerland. This investigation also undertook to identify the PRR involved in the sensing of *F. culmorum* by DC.

## MATERIALS AND METHODS

### Sampling of Fungi From Wheat Dust

Aerosols were collected on gelatin filters (25 mm, Sartorius AG, Gottingen, Germany) during wheat harvesting between July 15 and August 15, 2010, in 44 wheat fields in the Vaud region of Switzerland. Sampling was performed at a flow rate of 2 L/min using pocket pumps (MSA Escort Elf, Mine Safety Appliance Company, Pittsburgh, PA, or SKC pocket pump 210-1002, SKC, Inc., Eighty-four, PA) and clear styrene cassettes with 3 sections (25 mm diameter, SKC, Inc.). The pumps were placed on the combine harvester, 3 m above the soil surface. The temperature, barometric pressure, and relative air humidity were measured at each sampling site with a thermo-hygrometer and

barometer PCE-THB 40 (PCE Group Iberica, Albacete, Spain).

### DNA Extraction and *Fusarium* Species Identification

The gelatin filters were placed in 6 ml 0.9% NaCl, shaken at room temperature for 15 min, and centrifuged for 30 min at  $8500 \times g$ . The pellet was mechanically disrupted with a Tissue Lyser (Qiagen, Hombrechtikon, Switzerland) in the first buffer of the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH). Then DNA was extracted according to the manufacturer's instructions. Amplification of the four most frequent *Fusarium* species associated with FHB (*F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae*) was performed with validated species-specific primers pairs, respectively MGB-GRA, MGB-Cul, MGB-Ave, and Fp82 (Moradi et al., 2010). Polymerase chain reaction (PCR) reactions were performed using the peqGOLD Taq DNAPolymerase (PiqLab, Axon Lab AG, Baden-Dättwil, Switzerland),  $1 \times$  PCR Buffer S, and  $1 \times$  Enhancer Solution P using 5  $\mu$ l DNA. The amplification conditions were described in Moradi et al. (2010).

### Fungal Strain and Spores Suspension Preparation

*Fusarium culmorum* 2156 strain was kindly provided by the Agroscope, the Swiss Center of Excellence for Agricultural Research (Changins, Switzerland). *Fusarium culmorum* 2156 spores were grown on potato dextrose agar (PDA) plates (Oxoid, Basingstoke, UK) and cultured for 7 d at 23°C. Spores were collected by flushing PDA plates with  $3 \times 10$  ml of sterile phosphate-buffered saline (PBS). The solution was filtered through large-mesh gauze to eliminate residual agar and centrifuged 10 min at  $3500 \times g$ . The pellet was resuspended in PBS and spores were enumerated. Spore suspensions were used immediately or kept for 24 h at 4°C or 22°C. Each spore suspension was divided into two equal parts: One was left untouched (called total preparation), whereas the other was centrifuged for 10 min at  $3500 \times$

g to separate the supernatant (SN) from spores (SP), which were resuspended in an equal volume of PBS. Inactivated spores were obtained by heating fresh suspensions of *F. culmorum* 2156 for 10 min at 100°C or 2 h at 56°C or by treatment for 10 min with 4% paraformaldehyde (PFA) and extensive washings.

### Mycotoxin Extraction

Spores from three independent cultures were used. Mycotoxins were extracted from spores or supernatants using a methanol (MeOH)/Milli-Q water (70:30, v/v) solution by shaking for 10 min and sonication for 15 min with a Branson 5210 apparatus (Branson Ultrasonics S.A., Carouge, Switzerland). Extracts were filtered with a 10-kD, 15-ml, Vivaspin 4 Centrifugal Concentrator (Sartorius Stedim Biotech, Aubagne, France) and evaporated for 90 min using 10 psi nitrogen with a TurboVap LV system (Capiler LifeSciences, Mountain View, CA). The residue was dissolved in 950  $\mu$ l MeOH/water (20:80, v/v).

### LC-MS/MS Analyses

Analytical standards of DON, 3-ADON, 15-ADON, NIV, and ZEA at 100  $\mu$ g/ml in acetonitrile were purchased from Fluka (Sigma-Aldrich, St Louis, MO). Internal standards of U-[ $^{13}\text{C}_{15}$ ]-DON, U-[ $^{13}\text{C}_{17}$ ]-3-ADON, U-[ $^{13}\text{C}_{15}$ ]-NIV, and U-[ $^{13}\text{C}_{18}$ ]-ZEA at 25  $\mu$ g/ml in acetonitrile were obtained from ROMER Labs (Tulln, Austria). Mycotoxins were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a Varian 1200L mass spectrometer (Varian, Walnut Creek, CA). Twenty-five microliters of each sample, including  $^{13}\text{C}$  internal standards (each at 1  $\mu$ g/ml), was loaded onto an Ascensis Express F5 column (100  $\times$  3.0 mm, 2.7  $\mu$ m) joined to an Ascensis Express F5 Guard column (5  $\times$  3.0 mm, 2.7  $\mu$ m; SUPELCO Bellefonte, PA) using an automatic injector. Mycotoxins were eluted at a flow rate of 0.4 ml/min with a water/methanol mobile phase as follows (percent water/methanol): 0–5 min 80/20,

5–18.5 min 4/96, and 18.5 min 80/20. Eluents (LC-MS quality, Carlo Erba reagents, Val-de-Reuil, France) were buffered with 5  $\mu$ M ammonium acetate, pH 7.1. LC-MS/MS interface conditions for ionization of the mycotoxins in the negative mode were needle voltage –4500 V, nebulizing gas (compressed air) 51 psi, drying gas N<sub>2</sub> 270°C and 19 psi, and shield voltage –600 V. MS parameters and collision cell energies are given in Table 1. Standard curves were generated based on six different concentrations of pure mycotoxins. The limits of detection (LOD) of DON, 3-ADON, 15-ADON, NIV, and ZEA were calculated based on signal-to-noise (S/N) ratios of 3:1 and were 0.43, 1.91, 2.01, 1.75, and 0.67 pg/mg spore, respectively. The limits of quantification (LOQ) of DON, 3-ADON, 15-ADON, NIV, and ZEA were calculated based on S/N ratios of 10:1 and were 1.43, 6.36, 6.71, 5.84, and 2.24 pg/mg spore, respectively.

### Mice and Cells

Eight- to 10-wk-old female C57BL/6 mice weighing 20–25 g (Charles River Laboratories, L'Arbresle, France) were housed under specific-pathogen-free conditions. MyD88 and dectin-1 knockout mice (MyD88<sup>−/−</sup> and dectin-1<sup>−/−</sup>) (Taylor et al., 2007; Kawai et al., 1999) on a C57BL/6 background were obtained from Professor Shizuo Akira (Osaka University, Japan) and Professor Gordon Brown (University of Aberdeen, UK). All animal procedures were approved by the "Office Vétérinaire du Canton de Vaud," Lausanne,

Switzerland (authorization numbers 876.7 and 876.8) and performed according to our institution guidelines for animal experiments. Bone marrow cells were obtained from femurs and tibias and cultured for 6 d in IMDM medium (Invitrogen, San Diego, CA), pH 7.4, supplemented with 10% heat-inactivated fetal calf serum (FCS; Seromed, Berlin, Germany), 50  $\mu$ M 2-mercaptoethanol, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin (Invitrogen), and granulocyte-macrophage colony-stimulating factor to generate bone-marrow-derived DC (BMDC) or macrophage colony-stimulating factor to generate bone marrow-derived macrophages (BMDM) (Lugrin et al., 2013; Roger et al., 2007).

### Cell Stimulation With *F. culmorum*

Loosely adherent immature BMDC were recovered by flushing with Versene (Invitrogen). BMDM were detached by incubation for 10 min at 4°C with Versene. Cells were washed with IMDM, counted, and seeded in 96-well cell culture plates (Corning Costar, Sigma-Aldrich) at a density of  $2.5 \times 10^5$  cells per well. BMDC and BMDM obtained from three independent mice were pooled before seeding. Cells were exposed for 24 h to an equivalent multiplicity of infection (MOI, i.e., a spore/cell ratio) of 0.01, 0.1, 1, or 10 of *F. culmorum* 2156 preparations. As controls of cell responsiveness, cells were exposed to 10 ng/ml *Salmonella minnesota* ultrapure lipopolysaccharide (LPS; List Biologicals Laboratories, Campbell, CA),

**TABLE 1.** Mass Spectrometry Parameters Used for Mycotoxin Detection

| Mycotoxin              | Q1 (m/z) | Q3 (m/z) | Capillary voltage (V) | Collision energy (eV) |
|------------------------|----------|----------|-----------------------|-----------------------|
| NIV                    | 311      | 281      | −55                   | 10                    |
| <sup>13</sup> C NIV    | 326      | 295      | −53                   | 10                    |
| DON                    | 295.2    | 279      | −60                   | 10                    |
| <sup>13</sup> C DON    | 310.1    | 279      | −60                   | 10                    |
| 3-ADON                 | 336      | 306      | −55                   | 10                    |
| <sup>13</sup> C 3-ADON | 354      | 322      | −50                   | 9                     |
| 15-ADON                | 337      | 149.1    | −50                   | 12                    |
| ZON                    | 317      | 131      | −50                   | 32                    |
| <sup>13</sup> C ZON    | 335      | 140      | −55                   | 50                    |

Note. Q1: precursor ion; Q3: main product ion.

2  $\mu\text{g/ml}$  CpG motif containing oligonucleotide 1826 (CpG; InvivoGen, San Diego, CA), 10  $\mu\text{g/ml}$  polyinosinic:polycytidylic acid (poly(I:C); InvivoGen), and 10  $\mu\text{g/ml}$  *Saccharomyces cerevisiae* hot alkali-treated zymosan (InvivoGen). LPS, CpG, poly(I:C), and zymosan are TLR4, TLR9, TLR3, and dectin-1 agonists, respectively. Images from cell cultures were recorded at 40 $\times$  magnification using an Eclipse TS 100 inverted microscope (Nikon, Tokyo, Japan) coupled to a Digital Sight DS-Fi1 Nikon camera (Nikon).

### Cell Viability Assay

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and viability assay (Mombelli et al., 2011; Tawadros et al., 2013). Briefly, at the end of incubation with *F. culmorum* 2156, cells were incubated for 2 h with 1 mg/ml MTT. Medium was removed and replaced by 100  $\mu\text{l}$  solution containing 10% sodium dodecyl sulfate (SDS), 5 mM HCl, and 66% (v/v) 2-propanol to lyse cells and dissolve formazan crystals. The absorbance was read at 570 nm using a Synergy H1 microplate reader (BioTek, Luzern, Switzerland). On each 96-well cell culture plate, serial quantities ( $0.3 \times 10^4$ – $5 \times 10^5$ ) of cells left untreated were processed in parallel to establish a standard curve.

### Quantification of Cytokines and Chemokines

Tumor necrosis factor (TNF), interleukin (IL)-6, and IL-12p40 concentrations in cell-culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, UK) (Roger et al., 2011). A screening of mediators produced by *F. culmorum* 2156-infected BMDCs cells was performed with a mouse cytokine Bioplex assay (Bio-Rad, Hercules, CA) using Luminex technology (Luminex Corporation, Austin, TX) (Delaloye et al., 2009). Twenty-one mediators were tested: TNF, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-33, interferon (IFN)  $\gamma$ , interferon gamma-induced protein (IP-10;

CXCL10), KC (CXCL1), macrophage inflammatory protein (MIP)-1 $\alpha$  (CCL3), MIP-1 $\beta$  (CCL4), MIP-2 (CXCL2), MIP-3 $\alpha$  (CCL20), monocyte chemoattractant protein (MCP)-1 (CCL2), RANTES (CCL5), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and matrix metalloproteinase (MMP)-9.

### Statistical Analyses

Comparisons between groups were performed by an analysis of variance (ANOVA) followed by Student's *t*-test. Two-sided *p* values less than .05 were considered to indicate statistical significance. All analyses were performed using PRISM (GraphPad Software, Inc., La Jolla, CA).

## RESULTS

### Frequency of *Fusarium* Species in Bioaerosols From Wheat Harvesting

Aerosols were sampled during wheat harvesting in 44 wheat fields in the Vaud region of Switzerland. Species-specific PCR revealed that *F. culmorum*, *F. poae*, and *F. graminearum* were present in 54, 40, and 36% of aerosols, respectively. *Fusarium avenaceum* was not detected. Considering these frequencies, subsequent experiments were performed using *F. culmorum* 2156, a *Fusarium* strain highly aggressive on Swiss wheat cultivars, from the collections of the Swiss Center of Excellence for Agricultural Research (Agroscope, Changins).

### Cytotoxic Effect of *Fusarium culmorum* Spores on Dendritic Cells

Dendritic cells play a critical role in sensing and processing of inhaled microorganisms and release of immunomodulatory mediators upon triggering by fungi (Roy and Klein, 2012). To establish the conditions for immune cell stimulation by *F. culmorum* 2156, mouse DC (i.e., BMDC) were exposed to increasing concentrations of freshly prepared suspensions of *F.*

*culmorum* 2156 spores and used as an indicator for production of IL-12p40, a cytokine that is usually abundantly secreted by DC exposed to microbial products. *Fusarium culmorum* 2156 was incubated with BMDC at ratios ranging from 1 to 1,000 spores per 100 cells (i.e., MOI 0.01 to MOI 10). IL-12p40 concentrations in cell culture supernatants collected after 24 h were measured by ELISA. IL-12p40 production followed a concentration-dependent bell-shaped response curve, with concentrations increasing between MOI 0.01 to MOI 1 and markedly decreasing using MOI 10 of *F. culmorum* 2156. Similar results were obtained for TNF and IL-6 production by BMDC or when using macrophages such as BMDM (data not shown) as responding cells, indicating that the effect was not specific to IL-12p40 or BMDC. Taken together, these results suggested that high concentrations of *F. culmorum* 2156 spores exert a toxic effect on innate immune cells. To test this hypothesis, the viability of BMDC was measured using the MTT assay. A significantly reduced cell survival was observed only when BMDC were exposed to *F. culmorum* 2156 at MOI 10 ( $36 \pm 8\%$  of the signal obtained with BMDCs cultured in control medium; Table 2).

The observed cell death might be attributed to toxic molecules contained in, or secreted by *F. culmorum* spores. Hence, cell death induced by spore (SP) and supernatant (SN) fractions that were obtained after centrifugation of *F. culmorum* 2156 suspensions was monitored. Moreover, to assess whether nongerminated and germinated *F. culmorum* 2156 behaved differently, spore and supernatant fractions were prepared from either fresh suspensions of *F.*

*culmorum* 2156 or suspensions kept at 4°C or 22°C for 24 h before use (germination was confirmed by microscopic examination). Total, spore, and supernatant fractions exerted no marked cytotoxic effect on BMDC when used up to MOI 1 (Table 2, and data not shown). At MOI 10, the spore fraction reduced cell viability ( $39 \pm 8\%$  of the control) to an extent similar to the total preparation, whereas supernatants showed no cytotoxicity, suggesting that *F. culmorum* 2156 did not release cytotoxic mediators at effective biological concentrations under the conditions tested. Similar results were also obtained using nongerminated and germinated preparations of *F. culmorum* 2156.

Mycotoxins are present at high concentrations in agricultural dusts (Hardin et al., 2009). *Fusarium culmorum* produces trichothecene mycotoxins and the estrogenic metabolite ZEA, which are cytotoxic for mammalian cells in part because they inhibit protein synthesis (Bin-Umer et al., 2011; Zinedine et al., 2007; Behm et al., 2012). To determine whether *F. culmorum* 2156 spores release mycotoxins that might impact immune cell behavior, DON, 3-ADON, 15-ADON, NIV, and ZEA were extracted and quantified by LC-MS/MS from *F. culmorum* 2156 spore and supernatant fractions. DON was detected in supernatants ( $8 \pm 2.4$  ng/ml), 3-ADON in spores and supernatants ( $4 \pm 1.5$  pg/mg spores and  $2.9 \pm 1.2$  ng/ml supernatant), and ZEA in spores ( $12.3 \pm 6.6$  pg/mg spores). No other mycotoxins were identified in the different fractions. Taking into consideration the cytotoxic effects induced by *F. culmorum* 2156 at MOI 10, the following experiments were performed using *F. culmorum* 2156 preparations at MOI 0.01–1.

**TABLE 2.** Cytotoxic effects of *Fusarium culmorum* on BMDCs

|                   | Cell viability (% of signal in control medium) |             |              |
|-------------------|------------------------------------------------|-------------|--------------|
|                   | MOI 0.1                                        | MOI 1       | MOI 10       |
| Total preparation | $111 \pm 15$                                   | $96 \pm 5$  | $36 \pm 8^*$ |
| Spores            | $116 \pm 18$                                   | $104 \pm 4$ | $39 \pm 8^*$ |
| Supernatant       | $124 \pm 1$                                    | $128 \pm 4$ | $103 \pm 6$  |

\* $p < .05$ .

### Dendritic Cells (DC) Ingest *Fusarium* Spores and Produce Large Amounts of Cytokines and Chemokines

DC are professional phagocytic cells. Indeed, careful microscopic examination clearly showed that BMDC ingested *F. culmorum* 2156 spores (Figure 1). In order

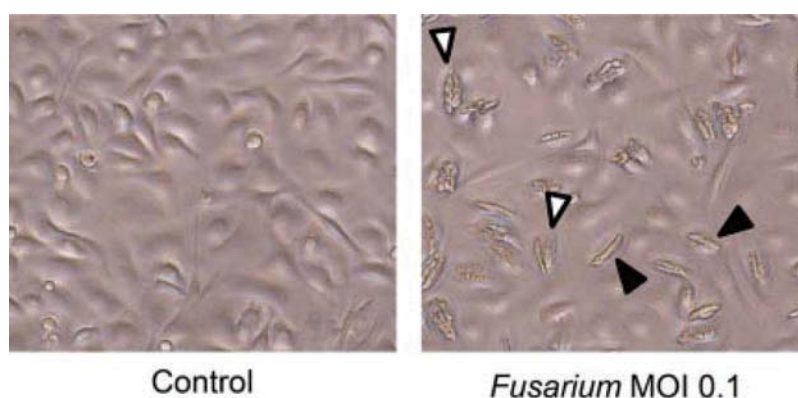


**FIGURE 1.** Ingestion of *Fusarium culmorum* spores by BMDCs. BMDCs were exposed for 24 h to freshly prepared *F. culmorum* 2156 spores equivalent to MOI 0 and 0.1. Images were recorded at 40 $\times$  magnification. Close and open arrows point to BMDCs having ingested one and two spores, respectively. Note that the compartments of macroconidia exhibit extensive swelling, indicating germination, whereas no germ tube are visible.

to determine whether *Fusarium* spores or its secreted products stimulated production of polarizing cytokines by BMDC, total, spore, and supernatant fractions were tested. As shown in Figure 2, the total and spore but not supernatant fractions of both nongerminated and germinated *F. culmorum* 2156 concentration-dependently stimulated release of high amounts of IL-12p40 by BMDC. Total suspensions induced quantitatively more IL-12p40 than spore suspensions when using germinated preparations, which may result from shedding of immunoreactive cell wall components or intracellular components during germination. Low levels of IL-12p40 were eventually produced by BMDC exposed to supernatant fractions, which was associated with a few contaminating spores observed by microscopy.

To determine whether *Fusarium* spores required being alive to stimulate BMDC, the IL-12p40-inducing activity of live, heat-inactivated (100°C for 10 min and 56°C for 2 h) and PFA-inactivated spores was compared with PFA-inactivated spores. The inactivation process fully abrogated spore germination (Figure 3A), and markedly affected IL-12p40 production (Figure 3B). Indeed, heat- and PFA-inactivated *F. culmorum* 2156 spores stimulated the release of 2- to 18-fold lower levels of IL-12p40 than live spores. Of note, *Fusarium* spores germinated with

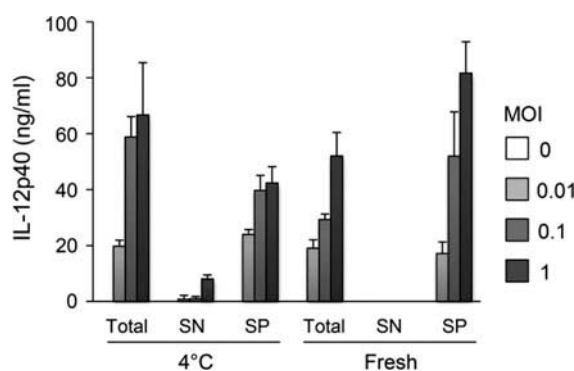


**FIGURE 2.** *Fusarium culmorum* spores induce the production of IL-12p40 by BMDCs. BMDCs were exposed for 24 h to increasing concentrations (equivalent to MOI 0, 0.01, 0.1, and 1) of *F. culmorum* 2156 fractions prepared as described in Materials and Methods. IL-12p40 concentrations were quantified by ELISA. Data are means  $\pm$  SD of triplicate samples from one experiment performed with a pool of BMDCs obtained from three mice. Similar results were obtained in a second experiment. Total: whole spore suspension; SN: supernatant; SP: spores; Fresh: fractions were prepared from fresh suspensions of *F. culmorum* 2156; 4°C: fractions were prepared from suspensions of *F. culmorum* 2156 kept for 24 h at 4°C in PBS. Significant at  $p = .02$  when comparing 4°C and fresh total vs. 4°C and fresh SP at MOI 0.1.

signs of swelling but essentially no germ tube production when incubated with BMDC (Figure 1). Thus, live spores appear to be more immunostimulatory than inactivated spores.

The cytokines produced by DC play a key role in shaping development of naive CD4 $^{+}$  T cells into Th1, Th2, or Th17 effector T cells that affect differentially antifungal

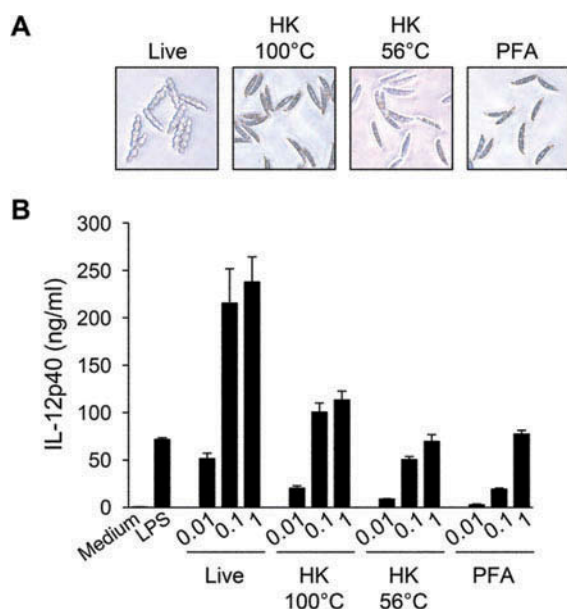


**FIGURE 3.** Inactivated *Fusarium culmorum* spores induce the production of reduced levels of IL-12p40 by BMDCs. (A) *Fusarium culmorum* 2156 spores were inactivated by heating (by incubation for 10 min at 100°C or 2 h at 56°C) or PFA treatment. Live and inactivated spores were cultured in IMDM for 24 h. Images were recorded at 40× magnification. BMDCs were exposed for 24 h to increasing concentrations (equivalent to MOI 0, 0.1, and 1) of freshly prepared live and inactivated spores or LPS (10 ng/ml). (B) IL-12p40 concentrations were quantified by ELISA. Data are means  $\pm$  SD of triplicate samples from one experiment performed with a pool of BMDCs obtained from 3 mice. HK: heat killed.

responses. Th1 and Th17 responses facilitate fungal clearance, whereas Th2 responses are involved in development of allergic reactions. To have a general overview of the mediators secreted by BMDC stimulated with *F. culmorum* 2156, the profile of cytokines (TNF, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-33, IFN $\gamma$ , GM-CSF, and G-CSF), chemokines (IP-10/CXCL10, KC/CXCL1, MIP-1 $\alpha$ /CCL3, MIP-1 $\beta$ /CCL4, MIP-2/CXCL2, MIP-3 $\alpha$ /CCL20, MCP-1/CCL2, and RANTES/CCL5) and MMP-9 was analyzed using the Luminex technology (Figure 4). This panel included cytokines involved in skewing or amplifying Th1 (IL-12p70, IFN $\gamma$ , TNF), Th2 (IL-4, IL-10), and Th17 (IL-6, CCL20) responses and powerful neutrophil chemoattractants (IP-10 and KC). Eleven of 21 mediators were detectable in cell-culture supernatants collected 24 h after

stimulation. As previously observed with IL-12p40 (Figure 2), total and spore preparations stimulated cytokine/chemokine release to a similar extent, while supernatants were mostly inactive. Interestingly, *F. culmorum* 2156 spores induced abundant quantities (20–90 ng/ml) of MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, MCP-1, and RANTES, and lower but still appreciable quantities (0.8–2.5 ng/ml) of TNF, IL-6, IL-12p70, IL-33, G-CSF, and IP-10.

### *Fusarium culmorum* Stimulates BMDCs Through Dectin-1 but Not MyD88

Dectin-1, TLR2, TLR4, and TLR9 are major receptors involved in the sensing of fungi by innate immune cells (Romani, 2011; Brown, 2011). All TLR except TLR3 trigger intracellular signaling and cytokine/chemokine production through recruitment of adaptor molecule MyD88 (Savva and Roger, 2013). The involvement of these TLR in immune responses may be indirectly monitored by assessing the impact of MyD88 deficiency. TLR3, but also TLR4, triggers signaling through the adaptor molecule TRIF involved in the production of type-I IFN and IFN-inducible genes (Savva and Roger, 2013). To investigate whether TLR and dectin-1 participated in the detection of *F. culmorum* 2156 by innate immune cells, the production of IL-12p40 by wild-type, MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, and dectin-1<sup>-/-</sup> BMDCs exposed was compared (Figure 5). The TLR4 and TLR9 agonists LPS and CpG were used as controls of MyD88 deficiency, the TLR3 agonist poly(I:C) as a control of TRIF deficiency, and zymosan ( $\beta$ 1,3-glucans) as a control of dectin-1 deficiency. *Fusarium culmorum* 2156 induced IL-12p40 release by wild-type and knockout BMDCs in a concentration-dependent manner. Dectin-1<sup>-/-</sup> BMDCs produced approximately twofold less IL-12p40 than wild-type BMDC, whereas MyD88<sup>-/-</sup> and TRIF<sup>-/-</sup> BMDC generated amounts of IL-12p40 similar to wild-type BMDC (Figures 5A, 5B, and 5C). Thus, evidence indicates that dectin-1 but not MyD88 or TRIF-dependent TLRs was necessary for recognition *F. culmorum* 2156 by BMDC.

## DISCUSSION

Results demonstrated that *F. culmorum* spores activate innate immune cells at least in part through a dectin-1-dependent pathway to release significant quantities of chemokines and pro-inflammatory cytokines, with a pattern of mediators more likely compatible with generation of Th1 or Th17 antifungal responses than with Th2 allergic responses. Interestingly, *F. culmorum* was the most represented of *Fusarium* spp. in aerosols sampled during wheat harvesting in the Vaud region of Switzerland, followed by *F. poae* and *F. graminearum*, whereas *F. avenaceum* was not detected. To our knowledge, no prior study analyzed the spectrum of *Fusarium* species in wheat dust aerosols using molecular techniques. However, the same *Fusarium* species were previously isolated using a culture-based method in a limited number of settled grain dust samples collected from farms in eastern Poland (Krysinska-Traczyk et al., 2001).

Exposure of DC and macrophages to high concentrations of *F. culmorum* 2156 spores (i.e., MOI 10) was associated with increased rate of cell death. Considering that *Fusarium* spp. may secrete mycotoxins with cytotoxic effects, the concentrations of mycotoxins expressed by *F. culmorum* 2156 were quantified by LC-MS/MS. These analyses revealed that DON was detected in supernatants, ADON in spores and supernatants, and ZEA in spores of *F. culmorum* 2156 preparations. Yet the concentrations of DON, 3-ADON, and ZEA were far lower than concentrations of mycotoxins previously reported to inhibit proliferation of innate immune cells in vitro. Indeed, the IC<sub>20</sub> of DON, 3-ADON, 15-ADON, and NIV ranged from 150 to 1500 ng/ml, whereas the IC<sub>50</sub> values of ZEA and its metabolites  $\alpha$ -zearalenol and  $\beta$ -zearalenol were approximately 3200 ng/ml against mouse macrophages (Pestka, 2008; Yu et al., 2011; Lu et al., 2013). With respect to DC, the IC<sub>50</sub> of NIV against BMDC was 200 ng/ml. BMDC were not sensitive to DON (up to 1200 ng/ml), contrary to porcine monocyte-derived DC (IC<sub>50</sub> DON = 800 ng/ml) (Luongo

et al., 2010; Bimczok et al., 2007). Although there are species- and cell-type-specific differences in the cytotoxic effects of mycotoxins, and mycotoxins derived from complex mixtures may exert synergistic effects (Alassane-Kpembi et al., 2013), it appears that the decreased viability of BMDC observed here was unrelated to production of DON, 3-ADON, 15-ADON, NIV, and ZEA by *F. culmorum* 2156. Although one cannot exclude that *F. culmorum* 2156 produced mycotoxins that were not analyzed in the present study (e.g., fusarenon-X), a more likely explanation is that innate immune cells underwent cell death due to overstimulation by fungal spores used at high MOI, as previously observed upon overstimulation of DC with microbial ligands (Zanoni et al., 2009). In agreement with this assumption, BMDC exposed to lower MOI released elevated amounts of cytokines.

Fungal toxins, particularly trichotecens, may possess immunomodulatory functions (Bondy and Pestka, 2000; Antonissen et al., 2014). However, our observations suggested that *F. culmorum* 2156 did not release mycotoxins in sufficient quantities to alter cytokine production by BMDC. Indeed, BMDC and BMDM exposed to nontoxic MOIs of *F. culmorum* 2156 produced large amounts of cytokines. Further, higher concentrations of DON, 3-ADON, and ZEA than the concentrations reached in our system were required to modulate cytokine production by human Jurkat T cells and porcine epithelial cells (Pestka et al., 2005; Taranu et al., 2014).

Activation of naive Th cells by DC requires three signals: (1) stimulation of T cells through recognition of antigenic peptides presented by major histocompatibility complex (MHC) molecules on DC by the T-cell receptor (TCR) expressed by T cells, (2) co-stimulation of T cells primarily by interaction of CD28 on T cells with CD80 or CD86 expressed by DCs, and (3) polarization of T cells by cytokines. Endocytosis is a prerequisite for antigen processing by antigen-presenting cells (APC) such as DC and macrophages. The fact that BMDC ingested *Fusarium* spores supports the hypothesis that APC present *Fusarium*-derived antigenic

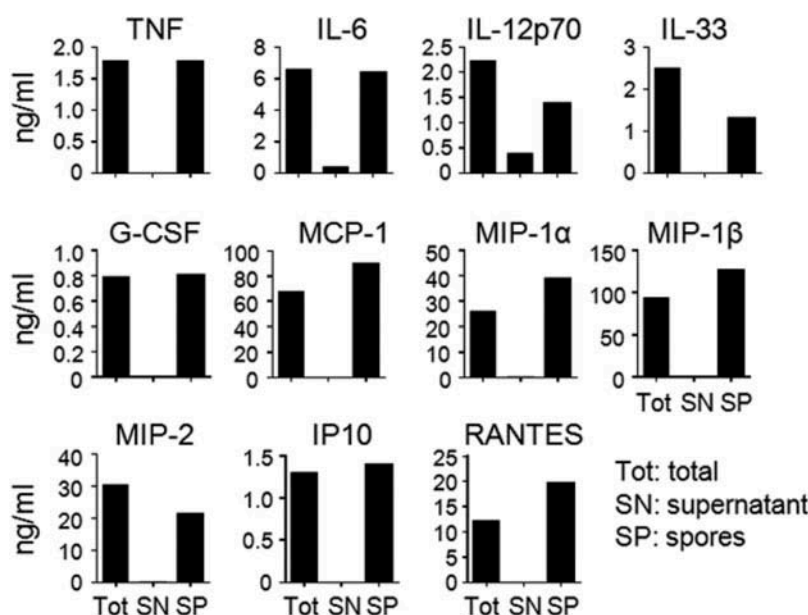


**FIGURE 4.** *Fusarium culmorum* spores induce the production of a large panel of cytokines and chemokines by BMDCs. BMDCs were exposed for 24 h to fresh preparations of *F. culmorum* 2156 used at an equivalent MOI 1. The concentrations of TNF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-33, IFNγ, IP-10 (CXCL10), KC (CXCL1), MIP-1α (CCL3), MIP-1β (CCL4), MIP-2 (CXCL2), MIP-3α (CCL20), MCP-1 (CCL2), RANTES (CCL5), MMP-9, GM-CSF, and G-CSF were determined by Luminex. Results are single measurements of the 11 mediators that were produced at detectable levels in cell culture supernatants upon *F. culmorum* 2156-stimulation. Tot: whole spore suspension; SN: supernatant; SP: spores.

peptides to T cells. Although analysis of whether DC increased expression of co-stimulatory molecules upon infection was not determined, this is likely occurring considering the potent immunogenicity of *Fusarium*. Indeed, innate immune response induced by *F. culmorum* in BMDC was characterized by marked chemokine production and a less abundant cytokine generation.

Overall, the pattern of cytokines and chemokines secreted by *F. culmorum*-infected BMDC suggested that BMDC favored antifungal protective immune responses rather than allergic responses, since IL-12p70, TNF, IL-6, and CCL20 were readily detected whereas IL-4 and IL-10 were not measurable. In agreement with our results, IFNγ and IL-17 expression was elevated and IL-4 not detected in human corneal tissues infected with *F. solani* (Karthikeyan et al., 2011). Grain workers frequently develop asthma or asthma-like symptoms. Asthma has long been considered as a Th2-mediated pathology, yet recent studies also involved Th17 cells in asthma development (Manni

et al., 2014). The mechanisms underlying predominant Th2, Th17, or Th2/Th17 mixed responses are still to be defined. Grain workers also suffer from chronic bronchitis-like symptoms. Chronic bronchitis develops after prolonged exposure to sufficient concentrations of fungal spores and is characterized by excessive inflammation and neutrophil influx in the airways. Although extrapolating our observations to pathologies prevalent in grain workers needs to be considered with caution, cytokines and chemokines induced by *F. culmorum* might be pathophysiologically relevant.

BMDC sensed *F. culmorum* 2156 at least partly through dectin-1, but not through MyD88 and TRIF-dependent pathways. β-Glucans, the primary dectin-1 agonists, are usually not exposed to surface of resting conidia, but the fungal cell wall is a dynamic structure changing during morphological transitions with influence on immune responses. For example, *Aspergillus fumigatus* swollen and germinating conidia stimulated more innate immune cells than dormant spores, which correlated

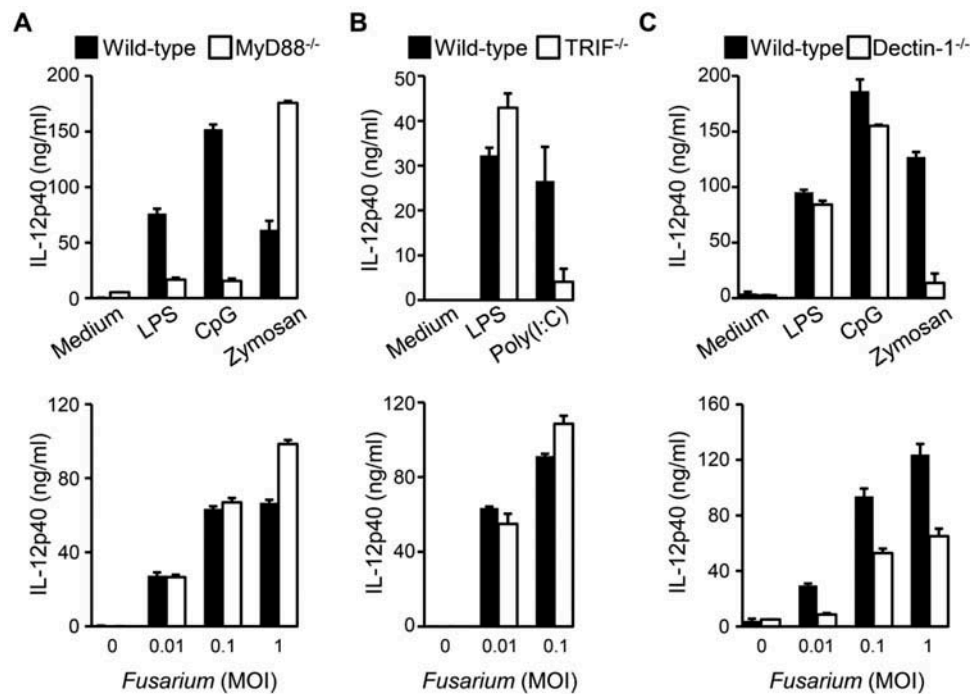


**FIGURE 5.** *Fusarium culmorum* stimulates IL-12p40 production by BMDCs in a dectin-1-dependent manner, but not MyD88 or TRIF-dependent manners. Wild-type and MyD88<sup>-/-</sup> BMDCs (A), wild-type and TRIF<sup>-/-</sup> BMDCs (B), and wild-type and dectin-1<sup>-/-</sup> BMDCs (C) were exposed for 24 h to LPS (10 ng/ml), CpG (2 µg/ml), poly(I:C) (10 µg/ml), zymosan (10 µg/ml), and increasing concentrations (equivalent to MOI 0, 0.01, 0.1 and 1) of freshly prepared *F. culmorum* 2156 spore suspensions. IL-12p40 concentrations were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with a pool of BMDMs obtained from three mice. Similar results were obtained in a second experiment (panels A and C).

with increased levels of surface-exposed  $\beta$ 1,3-glucans (Steele et al., 2005; Hohl et al., 2005). Unfortunately, nothing is apparently known about possible differences in the cell wall structure of resting and germinating *Fusarium culmorum* spores. Yet the cell wall of *Fusarium* spp contains  $\beta$ 1,3-glucans,  $\alpha$ 1,3-glucan, and chitin (Schoffemeer et al., 1999), and *F. culmorum* spores germinated during the culture process used in this study. Moreover, inactivation of *F. colmurum* spores using three different protocols abrogated spore germination and reduced spore-induced cytokine production by BMDC. It is conceivable that living *F. colmurum* spores are more immunogenic than inactivated spores because they expose  $\beta$ -glucans during germination.

Besides C-type lectin receptors, TLR are involved in recognition of diverse fungal components (Brown, 2011; Romani, 2011). Interestingly, TLR4 deficiency impaired neutrophil recruitment to corneal stroma

and worsened *Fusarium oxysporum*-induced keratitis (Tarabishy et al., 2008), indicating that different PRR are involved in the sensing of diverse *Fusarium* species or that PRR have cell-type/tissue specific roles. Differential PRR requirement may also condition the cytokine environment generated by DC and impact subsequent immune responses. Indeed, interaction of dectin-1 with TLR is required to amplify production of IL-1 $\beta$ , IL-6, and IL-23 and downregulate IL-12 in DC. This shift in cytokine response favors balance toward Th17 responses at the cost of Th1 responses (Vautier et al., 2010). The fact that the response of dectin-1<sup>-/-</sup> BMDCs to *F. culmorum* was not fully blunted suggests that additional PRR are involved in fungal detection. Among the possible candidates are complement receptor 3 and scavenger receptors (CD5, CD36, and SCARF1) that bind  $\beta$ -glucans, and mannose receptor, dectin-2, dectin-3, DC-SIGN, and mincle that sense mannans (Vacher et al.,

2014). It should also be mentioned that alveolar macrophages or lung DC might respond differently to *Fusarium* spores than BMDC and that species specificity might exist in host recognition of fungi (Rubino et al., 2012). Further studies are required to fully elucidate the pattern of PRR involved in the sensing of *F. culmorum* in the airways.

In summary, data showed that *F. culmorum* spores activate innate immune cells, at least in part through a dectin-1-dependent pathway, to release significant quantities of chemokines and proinflammatory cytokines. Although the pathophysiological relevance of this observation for grain worker diseases remains to be demonstrated, the cytokine/chemokine induced by *F. culmorum* may well play a role in the initial events that drive lung inflammation and promote airway pathologies upon grain dust inhalation. Further in vitro and in vivo investigations with additional fungal strains are required in order to have a better understanding of *F. culmorum* mycotoxin and allergen production, cytotoxicity, and immunomodulatory activities with a special emphasis on T helper responses and innate immune recognition by PRR other than dectin-1. In addition, epidemiologic and immunogenetic studies would greatly expand our understanding of possible associations between *Fusarium* species prevalence and genetic polymorphisms with lung diseases related to grain handling (Vacher et al., 2014). Broadening the understanding of the pathogenesis of organic dust-induced disorders may help to improve or develop diagnostic and treatment strategies.

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