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Aspergillus fumigatus conidia induce IFN-β signaling in respiratory epithelial cells[1]

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Abstract

Aspergillus fumigatus is a fungal pathogen of major clinical importance. However, little is known about the role of human bronchial epithelial cells (HBEC) during *A. fumigatus* conidia induced inflammation. Here, we show that differentiated respiratory epithelial cells recognize inactivated resting conidia but not swollen conidia or hyphae resulting in the induction of the IFN-β signaling pathway and the expression of IFN-β inducible genes such as IP-10. This induction was internalization dependent. We identified double stranded conidia RNA recognized by TLR-3 as a factor responsible for the expression of IFN-β and IP-10. Inhibition of RIP-1/TBK-1, known to mediate IFN-β signaling, was sufficient to inhibit the induction of IFN-β and IP-10 expression by conidia. Even though conidia induced the activation of NF-κB in HBECs, IP-10 expression was only partially dependent on NF-κB signaling. These results provide evidence that respiratory cells are activated by double stranded RNA of resting conidia and initiate a first immune response to inhaled conidia in an IFN-β dependent manner.

Keywords: *Aspergillus fumigatus*, epithelium, IFN-β, NF-κB

Introduction

A. fumigatus is a fungal pathogen of major clinical importance. Inhalation of fungal spores may cause lung diseases ranging from local inflammation of the airways to severe and life threatening infections of the lung such as allergic broncho-pulmonary aspergillosis or invasive aspergillosis (1;2). Even though every human being inhales around 100 *A. fumigatus* conidia every day, severe aspergillus infections are relatively rare in healthy individuals indicating efficient immune mechanisms against fungal pathogens (3).

The mucosal surface of the respiratory tract provides a physical barrier where inhaled conidia encounter defense mechanisms of the host for the first time. At the epithelial surface of the upper and lower respiratory tract, conidia are trapped in mucus and removed with the help of ciliated cells. In the lower respiratory tract, alveolar macrophages represent a first-line innate host defense mechanism for clearing inhaled *A. fumigatus* from the lung (4-6). It has been shown that in the alveolar space, conidia are taken up by resident macrophages and killed by reactive oxygen species (ROS) (5). In spite of the efficiency of alveolar macrophages in removing inhaled particles, conidia sometimes germinate to hyphae, which are also recognized by macrophages. For instance, it has been shown that maturing but not resting (non-germinated) *A. fumigatus* conidia stimulate NF-κB in macrophages in a partially TLR-2 dependent manner leading to the secretion of pro-inflammatory cytokines and the production of ROS (7). Furthermore, Steele et al. showed that the beta-glucan receptor dectin-1 is required for the pro-inflammatory responses of alveolar macrophages to swollen and germinating *A. fumigatus* conidia (6). Whether respiratory epithelial cells play an active role in the defense against invading fungal pathogens is not well understood. However, in recent years, it has become evident that the epithelial surface of the respiratory tract not only provides a physical barrier that is highly effective in blocking penetration by most microbes but also has the ability to recognize microbes and to initiate an immune response (8;9). Similar to classical immune cells such as macrophages or neutrophils, respiratory epithelial cells are known to express a variety of pattern recognition receptors including Toll-like and NOD-like receptors allowing for the detection of diverse pathogens such as bacteria and viruses (10). Even though innate immune functions of respiratory epithelial cells are well described in response to bacteria and viruses, little is known about the response of these cells to fungal pathogens such as *A. fumigatus* and its spores, the conidia. It has been shown that surfactant found in the fluids lining the epithelial surfaces of the

lower respiratory tract binds conidia (11;12) and therefore enhances their uptake by neutrophils and alveolar macrophages (13). In addition, it could be shown that airway epithelial cells can internalize conidia thereby serving as reservoirs for immune cell evasion and dissemination throughout the host (14). Furthermore, it has been shown that germinating but not resting (non-germinated) conidia induce the expression of interleukin-8 in the human bronchial epithelial cell line BEAS-2B (15).

It was the aim of this study to investigate whether differentiated respiratory epithelial cells recognize *A. fumigatus* conidia and initiate an immune response. We demonstrate that inactivated resting conidia activate epithelial cells in an internalization dependent manner resulting in the activation of the IFN-ß signaling pathway and that double stranded RNA derived from conidia is responsible for TLR-3/ RIP-1/TBK-1 signaling in epithelial cells.

Methods

Cell culture, medium, and reagents

HBECs were isolated from large airways resected during surgery and cultivated as submersed or air-liquid interface (ALI) cultures as described previously (16). The protocol was approved by the Institutional Review Board (ethics committee) of the University of Marburg, and informed consent was obtained from the patients. The experiments presented in this study were done with cells obtained from seven individual patients who underwent surgery because of lung cancer. Only macroscopically and microscopically cancer free tissue was used for cell isolation. *A. fumigatus* (clinical isolate 27707) was cultured on YPD-agar plates (15 µg/ml penicillin and 40 µg/ml streptomycin to prevent bacterial contamination). Conidia were obtained by flushing the *A. fumigatus* culture with PBS/0.1 % Tween20. The resting conidia obtained were washed with PBS three times and counted. For experiments with swollen conidia, conidia were incubated for 3 or 6 hours in RPMI 1640 at 37°C. For experiments with hyphae, conidia were incubated in RPMI 1640 for 48 hours and the grown hyphae were dried. The dry, weighed hyphae were suspended in PBS to obtain a solution of 50 mg/ml. Resting conidia, swollen conidia and hyphae were heat inactivated (90°C for 60 minutes) or inactivated by UV-light and a sample was cultured over night to confirm complete inactivation. HBEC cytotoxicity was determined using an LDH-Cytotoxicity Assay Kit according to the manufacturer's instructions (Abcam, Germany).

Stimulation of HBEC with conidia and hyphae

HBEC cultures were stimulated apically with indicated concentrations of conidia and hyphae. In some cases, HBECs were incubated for 1 hour with the indicated concentrations of Cytochalasin D (Zygosporium mansonii; EMD, USA), resveratrol (EMD Biosciences, USA), or helenalin (EMD, USA) before stimulation. Experiments were repeated at least twice and run in triplicates.

RNA isolation and realtime RT-PCR

Total RNA of cells and conidia was isolated using the RNeasy Mini kit (Qiagen, Germany). For conidial RNA isolation, 1 x 10^9 conidia were frozen in liquid nitrogen and ground in a mortar before RNA was isolated (1.5 µg RNA / 10^9 conidia). Where indicated, conidia RNA was digested with RNase I or RNase III (New England Biolabs, Germany). RNA was reverse transcribed using a cDNA synthesis kit (MBI Fermentas, Germany). A SYBR-Green-PCR mix

(ABgene, Germany) and beta-actin primers (sense, 5'-AGCCTCGCCTTTGCCGA -3'; antisense, 5'-CTGGTGCCTGGGGCG -3'), IFN-ß primers (sense, 5'-CAGCAATTTTCAGTGTCAGAAGC-3'; antisense, 5'- TCATCCTGTCCTTGAGGCAGT-3'), and IP-10 primers (sense, 5'-TGAAATTATTCCTGCAAGCCAA-3'; antisense, 5'-CAGACATCTCTTCTCACCCTTCTTT-3') were used. Quantitative PCR results were obtained using the ΔΔCT method. Experiments were repeated at least twice and run in triplicates.

Determination of cytokine concentrations

Levels of IP-10, IFN-β, and IL-8 in cell-free supernatants were determined by commercially available sandwich-type ELISA (R&D Systems, Germany).

Transfection experiments

Transfections were performed using TransIT LT-1 (Mirus Bio Corporation, USA). For transfection of HBECs with conidia RNA, $1x10^5$ cells were seeded in a 12-well plate. For NF-κB activation experiments, $5x10^4$ HBECs cultivated in 24-well plates were transfected with 0.5 µg 5xNF-κB-luciferase-reporter plasmid per well. The medium was removed after 24 hours and medium containing conidia or 10 ng/ml TNF-α as control was added for another 24 hours. Luminescence was measured using a Dual-Luciferase Reporter Assay System (Promega,). HEK-293 cells were seeded in a six well plate and transfected with 1 µg hTLR-3-plasmid or 1 µg pcDNA3.1-plasmid.

Statistical analysis

Values are displayed as mean ± SEM. Comparisons between groups were analyzed by t test (two-sided), or ANOVA for experiments with more than two subgroups. Post hoc range tests were performed with the t test (two sided) with Bonferroni adjustment. Results were considered statistically significant for $p < 0.05$.

Results

A. fumigatus conidia induce internalization dependent IFN-β and IP-10 expression in respiratory epithelial cells

Inhaled *A. fumigatus* conidia can germinate, develop hyphae, and cause severe disease, especially in immuno-compromised individuals (2;3). To characterize the inflammatory response of respiratory epithelial cells to different developmental stages of *A. fumigatus* differentiated, primary HBECs grown as ALI cultures and as conventional liquid cultures were stimulated with heat or UV-light inactivated resting (non-germinated) conidia, swollen (germinated) conidia, and hyphae of *A. fumigatus*. Interestingly, there was a strong and dose dependent upregulation of IFN-β and IP-10 expression in HBECs when stimulated with heat and UV-light inactivated resting conidia that could not be observed when the cells were incubated with swollen conidia or hyphae as shown by realtime RT-PCR analysis (Fig. 1 A/B). Furthermore, figure 1C shows that the induction of IFN-β precedes the induction of IP-10 reaching statistical significance after 4 and 24 hours, respectively.

It has been shown that conidia are internalized by ciliated and non-ciliated epithelial cells and end up in the endosomal pathway (14;17-19). To examine whether the observed induction of IFN-β and IP-10 requires internalization of conidia by epithelial cells HBECs were pre-incubated with the endocytosis inhibitor cytochalasin D and stimulated with heat inactivated resting conidia. The induction of IFN-β and IP-10 expression in HBECs incubated with heat inactivated resting conidia was inhibited by cytochalasin D in a dose dependent manner (Fig. 2 A). In addition, ELISA analysis revealed that the release of IP-10 into the culture supernatant was also inhibited by cytochalasin D (Fig. 2 B, the levels of IFN-β were below the ELISA detection limit). An LDH release assay showed that the observed effects were not due to cytotoxicity (data not shown).

Together, these results demonstrate that resting (non-germinated) conidia but not germinated conidia or hyphae activate primary airway epithelial cells resulting in the activation of the IFN-β signaling pathway and the release of IFN-β inducible proteins.

HBEC recognize double stranded conidia RNA

Next, we sought to determine which factors of the conidia mediate the induction of IFN-β and IP-10. As the activation of the IFN-β signaling pathway had been associated with foreign RNA before (20) we isolated RNA from conidia and transfected it into HBECs. The conidia RNA induced IFN-β and IP-10 expression at similar levels than the heat and UV-light inactivated resting conidia whereas external RNA given directly into the culture medium did not induce expression of IFN- β or IP-10 (Fig. 3A). To evaluate whether single stranded RNA (ssRNA) or double stranded RNA (dsRNA) is stimulatory for HBECs native conidia RNA was digested with RNase I specific for ssRNA or RNase III specific for dsRNA. Treatment of conidia RNA with RNase III resulted in a complete loss of its stimulatory activity after transfection whereas RNase I had no effect (Fig. 3B). These results show that dsRNA of conidia activates IFN-ß signaling in HBECs.

The induction of IFN-β and IP-10 is RIP-1/TBK-1 dependent

It is known that IFN-β and IP-10 expression is dependent on the adaptor-protein TRIF that is, for instance, involved in the TLR-3 signaling cascade (21). Furthermore, the kinases RIP-1 and TBK-1 are essential factors of the TRIF signaling cascade (22) and can be inhibited with resveratrol (23). To examine whether the observed induction of IFN-β and IP-10 expression requires RIP-1 and TBK-1 activity, HBECs were pre-incubated with resveratrol and stimulated with heat inactivated resting conidia. The induction of IFN-β and IP-10 expression (Fig. 4 A) as well as the release of IP-10 into the culture supernatant (Fig. 4B) was inhibited by resveratrol in a dose dependent manner.

The transcription factor NF-κB is known to be activated within the TLR signaling cascade and is involved in the expression of many of inflammatory cytokines (24;25). However, it has been shown that the TRIF and IRF3 mediated expression of IFN-β does not depend on the activation of the NF-κB signaling cascade and that TLR-3 activation by double stranded RNA results only in a delayed activation of NF-κB (22). To test whether conidia induce NF-κB activation HBECs were transfected with a plasmid expressing luciferase under the control of a promoter containing NF-κB binding sites. Fig. 5A shows that incubation of HBECs with heat inactivated resting conidia (10^6 conidia per ml) or TNF-α, serving as positive control, resulted in expression of luciferase indicating that NF-kB is activated by conidia. However, inhibition of NF-κB with the

NF-κB inhibitor helenalin resulted only in a partial inhibition of the induction of IP-10 expression by conidia whereas IFN-β expression was not affected at all (Fig. 5B). To determine whether conidia induce the release of factors known to be under control of the NF-κB signaling cascade we measured the release of IL-8 into the supernatant by ELISA. Only high doses of conidia (10^6 and 10^7 conidia per ml) induced a modest release of IL-8 by HBECs (Fig. 5C). Together, these results show that RIP-1/TBK-1 signaling is involved in the activation of IFN-ß signaling in HBECs stimulated by conidia whereas NF-κB signaling does not play a major role in the signaling cascade mediating the expression of IFN-β and IP-10.

TLR-3 is activated by double stranded conidia RNA

To test whether TLR-3 contributes to the detection of dsRNA of conidia HEK-293 cells were transfected with a plasmid containing human TLR-3 and incubated with native conidia RNA as well as conidia RNA digested with RNase I or RNase III. HEK-293 cells expressing hTLR-3 showed a strong induction of IFN-β and IP-10 expression when stimulated with native RNA or dsRNA (RNase I treated RNA) compared to HEK-293 cells transfected with a control plamid (Fig. 6). Treatment of conidia RNA with RNase III before stimulation resulted in a complete loss of the stimulatory activity. Poly I:C, a specific ligand for TLR-3, served as positive control.

Discussion

This study examined the inflammatory response of differentiated airway epithelial cells to inactivated resting *A. fumigatus* conidia. The main finding is that airway epithelial cells are activated by resting conidia, but not by swollen conidia and hyphae, resulting in the induction of inflammatory mediators (IFN-β and IP-10) known to connect the innate and adaptive immune response of the host (26;27). It is shown that conidia recognition involves the TLR-3/ TBK-1/RIP-1 signaling cascade and that double stranded RNA is the pattern recognized by differentiated epithelial cells. These results give new insights into how airway epithelial cells contribute to the immune response of the host to inhaled resting conidia and are different from mechanisms described before for germinated conidia and hyphae (15;28-30).

Fungal spores are potent allergens and associated with allergic and chronic diseases of the respiratory tract such as asthma (31). Moreover, inhaled conidia may cause life-threatening infections of the lung such as allergic broncho-pulmonary aspergillosis or invasive aspergillosis in immune compromised individuals. It is obvious that the host needs to clear inhaled conidia to prevent their germination and growth. Even though inhaled particles are trapped in the fluids and mucus lining the epithelial surfaces of the upper respiratory tract and are removed with the help of ciliated cells, it is evident that epithelial cells of the respiratory tract need to be actively involved in initiating and modifying the innate and adaptive immune response as it is known for bacterial and viral pathogens. Indeed, former studies showed that ciliated and non-ciliated respiratory epithelial cells as well as the bronchial epithelial cell line 16HBE14o- internalize *A. fumigatus* conidia (17;19;32;33). In addition, it has been shown in the epithelial cell line A549 and in primary respiratory epithelial cells that internalized conidia are directed into the endosomal-lysosomal compartment where they are acidified and partially killed (18;33). Furthermore, it has been shown that the interaction of the respiratory epithelial cell line BEAS-2b with germinating but not resting (non-germinated) conidia results in the induction of pro-inflammatory cytokines such as IL-8. Balloy et al. showed that this induction is mediated via a signaling cascade involving phosphatidylinositol-3-kinase, p38 MAPK, and ERK1/2 independent of the MyD88 pathway (15). These results are in line with a study showing

that A. *fumigatus* induces innate immune responses in alveolar macrophages via MAPK signaling pathways independent of TLR2 and TLR4 and that recognition by TLR2/4 and MyD88 signaling are dispensable for the clearance of *A. fumigatus* in immuno-competent individuals (29). In contrast, Mambula et al. demonstrated that TLR2 and MyD88 mediate a response of macrophages to live resting conidia of *A. fumigatus* shown by the induction of TNF-α (30). Furthermore, a recent study showed that conidia of *Histoplasma capsulatum* induce IFN-β expression in murine macrophages in an IRF3 dependent manner (34). Our finding that heat or UV-light inactivated and thus metabolically inactive resting conidia are recognized by differentiated HBECs resulting in the expression of the inflammatory mediators IFN-ß and IP-10 offers new insights into the underlying signaling pathways of *A. fumigatus* recognition at an early state of fungal infection. Our study adds to the studies mentioned above that used live conidia as well as germinated conidia and hyphae with focus on pro-inflammatory cytokines such as IL-8 and TNF-α and the TLR2/4-MAPK-signaling pathways (29;30). The results presented in this study suggest TLR-3 as receptor contributing to the activation of respiratory epithelial cells by recognizing double stranded RNA of conidia. Our findings are in line with a study published recently showing that epithelial cells provide protection in the lungs of mice infected with *A. fumigatus* conidia in a TLR3/Trif-dependent manner (35). Former studies also showed that TLR-3 is expressed in primary respiratory epithelial cells and that TLR-3 signaling is associated with interferon signaling pathways (10). TLR-3 is described to be localized in endosomal membranes where it recognizes double stranded RNA (25;36). This is in line with our finding that the activation of HBECs was dependent on endocytosis of the conidia. Furthermore, isolated conidia RNA was only able to induce IFN-ß and IP-10 expression when it was transfected into HBECs, whereas no induction of IFN-ß and IP-10 could be observed when the RNA was given directly to the culture medium. In addition, isolated conidia RNA strongly induced IFN-ß and IP-10 in HEK-293 cells over-expressing hTLR-3 but not in control HEK-293 cells. However, there are additional recognition mechanisms known for double stranded RNA involving cytosolic protein kinase R (PKR) (37) and RNA-helicases such as RIG-I and mda-5 (38;39). A contribution of these receptors to the activation of HBECs by conidia cannot be completely excluded. In fact, for transfected RNA it is reasonable to suggest that cytosolic receptors for double stranded RNA mediate the

induction of IFN-ß and IP-10 independent of TLR-3 as transfected RNA gets directly into the cytosol of HBEC. Our finding that inhibition of RIP1 and TBK1 blocked the induction of IFN-ß and IP-10 by inactive conidia excludes PKR as receptor of double stranded conidia RNA as RIP1 and TBK1 are involved in TLR-3 and RIG-1/mda-5 signaling but not in PKR signaling (40). TBK1 is essential for the induction of IRF3 regulated genes such as IFN-ß (21) whereas phosphorylation of RIP-1 is involved in the late activation of NF-κB (24). In addition, our results show that only the expression of IP-10 is partially dependent on NF-κB signaling. This is in line with studies showing that the induction of IFN-β via the TLR-3 signaling cascade does not rely on the activation of NF-kB even though TLR-3 signaling leads to a late activation of NF-kB (36). As only double stranded RNA showed stimulatory activity in this study, we suggest that double stranded RNA derived from mycoviruses and conidial mRNA are responsible for the induction of IFN-β signaling in epithelial cells. Former studies showed that mycoviruses are efficiently transmitted by asexual spores containing up to 120 µg double stranded RNA per gram dry weight and that double stranded RNA derived from fungal mycoviruses is a potent inducer of interferon (41-44). Furthermore, mRNA has been shown to be a potent ligand of TLR-3 likely due to secondary double stranded structures (45).

Besides its role in the defense of viruses, IFN-ß plays an important role in the differentiation and activation of effector cells of the innate and adaptive immune system such as macrophages, dendritic cells, and T-cells (46). IFN-ß inhibits apoptosis of T-cells and enhances the expression of co-stimulatory factors such as CD40 and CD86 in dendritic cells (47). Another function of IFN-ß is the induction of a group of genes called IRG (interferon response genes) including IP-10 (48) thereby linking innate and adaptive immunity (49). IP-10 is chemotactic for monocytes/macrophages, T-cells, and dendritic cells (26). It is reasonable to conclude that the expression of IFN-ß and IP-10 by epithelial cells plays an important role in directing monocytes/macrophages and other professional immune cells to the respiratory tract to ensure efficient clearance of inhaled and germinating spores. In addition, IP-10 is described to contribute to allergic pulmonary inflammation and airway hyperreactivity (27). Thus, epithelial derived IP-10 may contribute to the progression of diseases such as allergic broncho-pulmonary aspergillosis.

Our data are in contrast to a study of Aimanianda et al. that demonstrated that the hydrophobic surface layer of non-swollen conidia prevents immune recognition of fungal spores (50). We suggest that internalization as well as lysosomal acidification and killing of conidia, as outlined above and in the results section, might be a mechanism by which double stranded RNA is released from the conidia when entering the endosomal pathway in epithelial cells.

In conclusion, our study provides insight into the mechanisms allowing respiratory epithelial cells to react to resting *A. fumigatus* conidia. Double stranded RNA of conidia induces the activation of the IFN-β signaling pathway and the expression of chemokines such as IP-10 indicating an important role of the epithelium in initiating an early immune response to inhaled conidia.

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Footnotes

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[*] Authors contributed equally

Figure legends

Fig. 1. Resting conidia but not swollen conidia or hyphae induce IFN-β and IP-10 expression in epithelial cells grown as submerged monolayer or as air-liquid interface (ALI) cultures. (A/B) Epithelial cells were inoculated for 24 h with the indicated concentrations of heat inactivated resting conidia, with 10^6/ml heat inactivated swollen conidia (germinated for 3 and 6 hours), with 100 µl of heat inactivated hyphae solution (50mg/ml), and with 10^6/ml UV-light inactivated resting conidia . (C) Submerged monolayers were inoculated with 10^3/ml heat inactivated resting conidia for the indicated times. Epithelial RNA was isolated and qRT-PCR was performed to measure the induction of IFN-ß (grey bar) and IP-10 (black bar). Results are shown as mean ± S.E.M. of at least two different experiments each performed in triplicate and using cells from different donors, * $P < 0.05$.

Fig. 1A

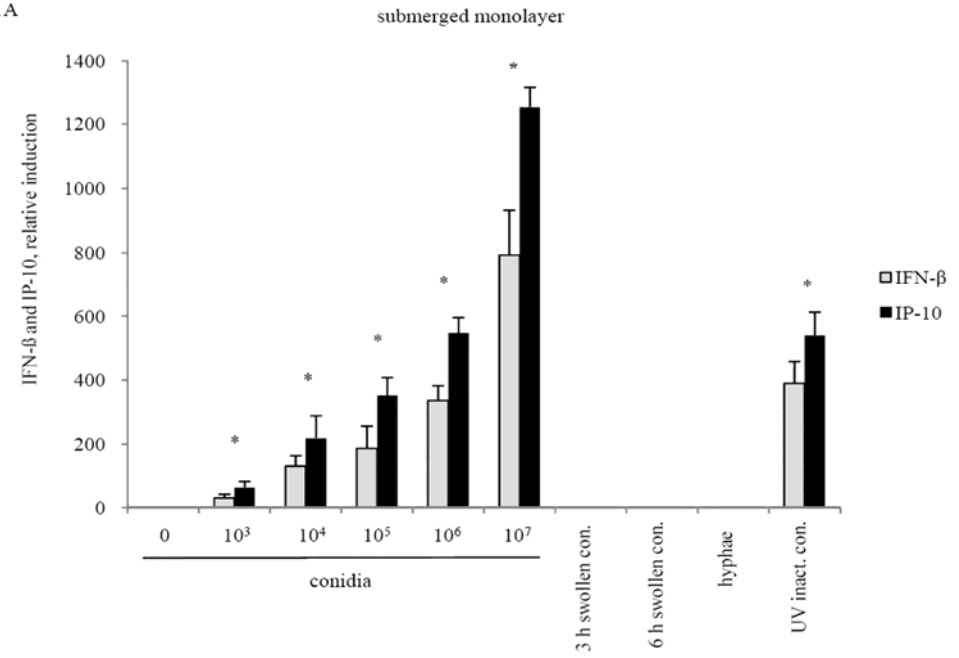

submerged monolayer
IFN-ß and IP-10, relative induction
1400
1200
1000
800
600
400
200
0
*
0
10³
10⁴
10⁵
10⁶
10⁷
conidia
3 h swollen con.
6 h swollen con.
hyphae
UV inact. con.
IFN-β
IP-10

Fig. 1C

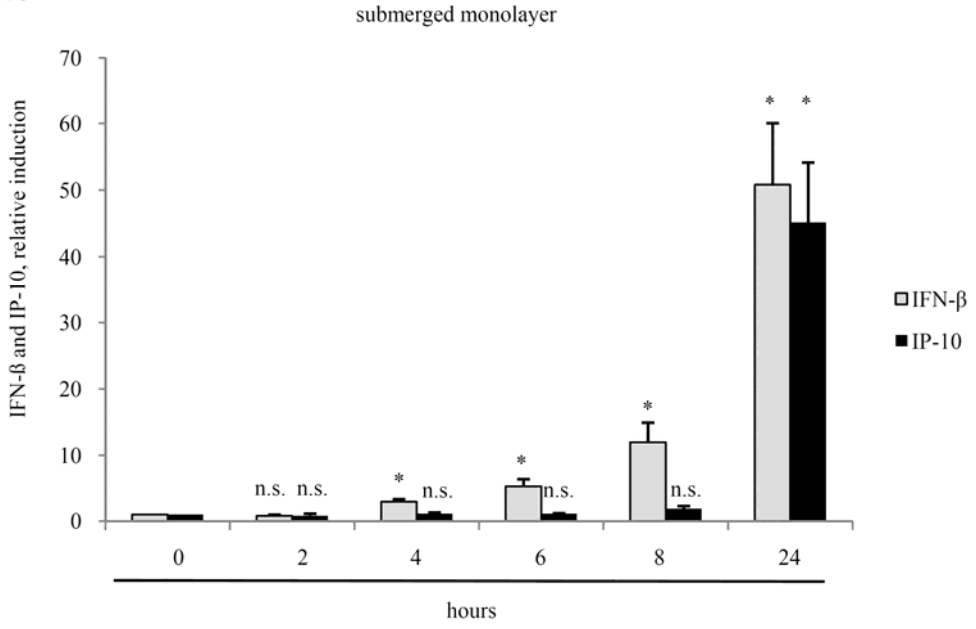

submerged monolayer
IFN-ß and IP-10, relative induction
70
60
50
40
30
20
10
0
n.s.
n.s.
n.s.
n.s.
n.s.
*
*
*
*
*
0
2
4
6
8
24
hours
IFN-β
IP-10

Fig. 2. Cytochalasin D inhibits the induction of IFN-β and IP-10. Submerged HBEC monolayers were inoculated with 10^6/ml heat inactivated resting conidia after pre-incubation with the indicated concentrations of cytochalasin D for 1 h. (A) At 24 h post inoculation, epithelial RNA was isolated and qRT-PCR was performed to measure the induction of IFN-ß (grey bar) and IP-10 (black bar). (B) IP-10 concentrations in supernatants were analyzed by ELISA. Results are shown as mean ± S.E.M. of at least two different experiments each performed in triplicate and using cells from different donors, *$P < 0.05$.

Fig. 2B

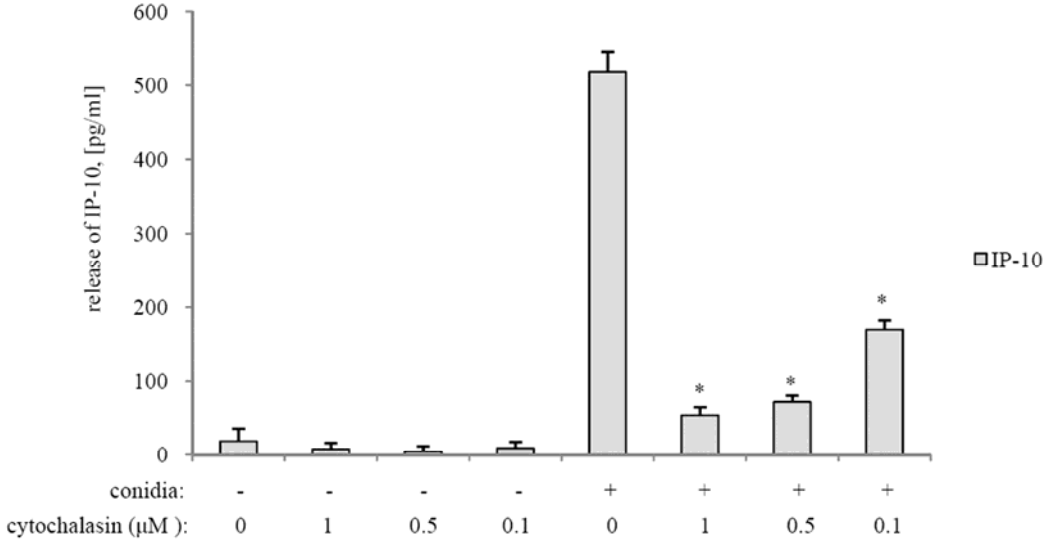

600
500
400
300
200
100
0
release of IP-10, [pg/ml]
IP-10
*
*
*
conidia: - - - - + + + +
cytochalasin (µM): 0 1 0.5 0.1 0 1 0.5 0.1

Fig. 3. Airway epithelial cells recognize double stranded conidia RNA. Submerged HBEC monolayers were transfected with the indicated concentrations (ng/ml) of RNA isolated from conidia or stimulated externally with conidia RNA given directly into the culture medium (A) or transfected with 200 ng/ml conidia RNA digested with RNase I or RNase III (B). At 24 h post inoculation, epithelial RNA was isolated and qRT-PCR was performed to measure the induction of IFN-ß (grey bar) and IP-10 (black bar). Results are shown as mean ± S.E.M. of at least two different experiments each performed in triplicate and using cells from different donors, * $P < 0.05$.

Fig. 3B

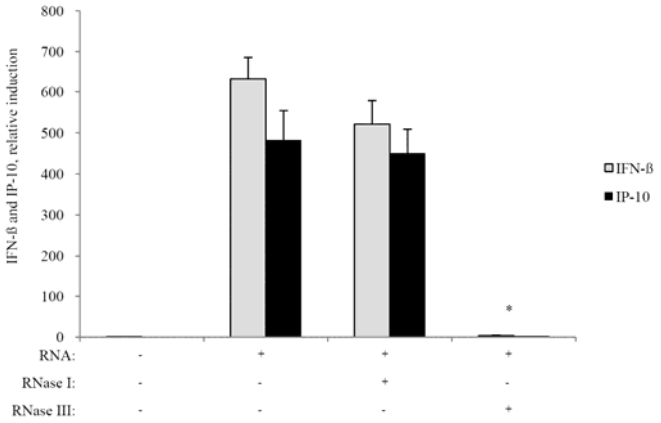

800
700
600
500
400
300
200
100
0
IFN-ß and IP-10, relative induction
*
IFN-ß
IP-10
RNA: - + + +
RNase I: - - + -
RNase III: - - - +

Fig. 4. Resveratrol inhibits the induction of IFN-β and IP-10. Submerged HBEC monolayers were inoculated with 10^6/ml heat inactivated resting conidia and incubated with the indicated concentrations of resveratrol. (A) At 24 h post inoculation, epithelial RNA was isolated and qRT-PCR was performed to measure the induction of IFN-ß (grey bar) and IP-10 (black bar). (B) IP-10 concentrations in supernatants were analyzed by ELISA. Results are shown as mean ± S.E.M. of at least two different experiments each performed in triplicate and using cells from different donors, * $P < 0.05$.

Fig. 4B

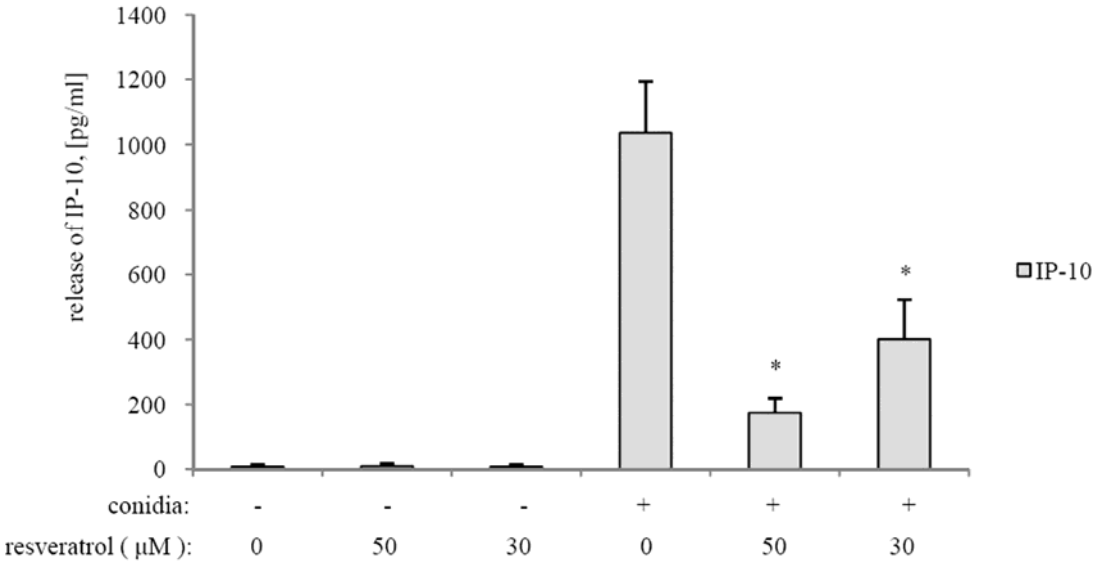

1400
1200
1000
800
600
400
200
0
release of IP-10, [pg/ml]
IP-10
*
*
conidia: - - - + + +
resveratrol (µM): 0 50 30 0 50 30

Fig. 5. Conidia activate NF-κB. Submerged HBEC monolayers were transfected with a plasmid expressing luciferase under the control of a promoter containing NF-κB binding sites (A) or incubated with the indicated concentrations of helenalin (B) and incubated with 10^6/ml heat inactivated resting conidia (A/B) or 10 ng/ml TNF-α (A). At 24 h post inoculation, cells were lyzed, substrate was added, and the relative light units (RLU) were determined (A) or epithelial RNA was isolated and qRT-PCR was performed to measure the induction of IFN-ß (grey bar) and IP-10 (black bar) (B). IL-8 concentrations in the supernatants of HBEC monolayers stimulated with the indicated concentrations of heat inactivated resting conidia or with 10 ng/ml TNF-α were analyzed by ELISA (C). Results are shown as mean ± S.E.M. of at least two different experiments each performed in triplicate and using cells from different donors, * $P < 0.05$.

Fig. 5C

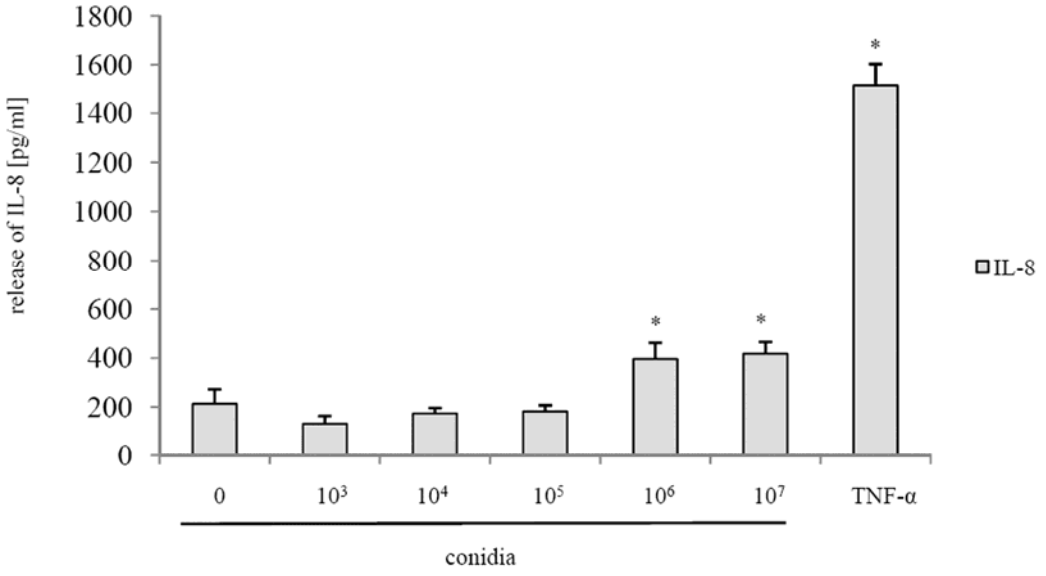
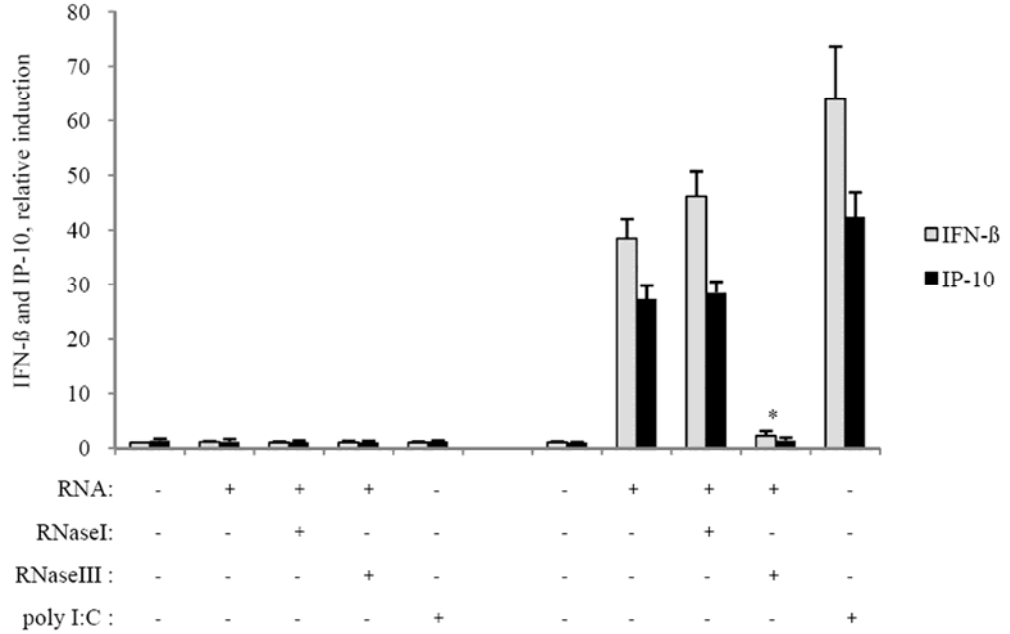

1800
1600
1400
1200
1000
800
600
400
200
0
release of IL-8 [pg/ml]
*
*
*
0
10³
10⁴
10⁵
10⁶
10⁷
TNF-α
conidia
IL-8

Fig. 6. TLR-3 recognizes double stranded conidia RNA. HEK-293 cells were transfected with a plasmid expressing human TLR-3 or a control plasmid and incubated with culture medium containing 5 μg/ml poly I:C or 200 ng/ml RNA isolated from conidia, either non-treated, digested with RNase I, or digested with RNase III. At 24 h post inoculation, HEK-293 RNA was isolated and qRT-PCR was performed to measure the induction of IFN-ß (grey bar) and IP-10 (black bar), * $P < 0.05$.

Fig. 6


IFN-ß and IP-10, relative induction
80
70
60
50
40
30
20
10
0
*
IFN-ß
IP-10
RNA: - + + + - - + + + -
RNaseI: - - + - - - - + - -
RNaseIII : - - - + - - - - + -
poly I:C : - - - - + - - - - +