Interaction analyses of human monocytes co-cultured with different forms of *Aspergillus fumigatus*

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Monocytes play a major role in the cellular defence against *Aspergillus fumigatus* in immunocompromised patients. To obtain a better understanding of the mechanisms involved in this interaction, phagocytosis and gene expression profiling of human monocytes was carried out after incubation with *A. fumigatus* resting, swollen and germinating conidia and hyphae (for 3, 6 and 9 h). The majority of monocytes phagocytosed up to three conidia during the first 3 h of incubation. Microarray analysis showed an increased expression level of immune-relevant genes, which was dependent on the germination state of the fungus and the incubation period. Among these genes, those encoding interleukin-8, macrophage inflammatory protein 3- α (CCL20) and monocyte chemoattractant protein-1 (CCL2) were found to be potential key regulators involved in the *A. fumigatus*-induced immune response. In addition, *A. fumigatus* was found to be an inducer of the genes encoding urokinase type plasminogen activator (uPA), urokinase type plasminogen activator receptor (uPAR), plasminogen activator inhibitor (PAI), pentraxin-3 (PTX3) and intercellular adhesion molecule-1 (ICAM-1), which, in combination, may contribute to thrombosis and local lung tissue injury.

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INTRODUCTION

Immunocompromised patients, such as patients with acute leukaemia or patients after solid-organ or stem-cell transplantation, are at high risk of severe and even fatal infections. One of the major pathogens is the opportunistic fungus *Aspergillus fumigatus*. The mortality rate in patients suffering from invasive aspergillosis (IA) can be up to 100 % (Denning, 1996; Wald *et al.*, 1997).

Activation of innate immune cells is crucial for successful control of IA. Phagocytes play an important role in the cellular defence against this fungus by attacking the fungus

itself, as well as by the release of pro- and anti-inflammatory cytokines and chemokines (Liu & Pope, 2004).

Alveolar macrophages are the main pulmonary phagocytic cells and are thus the first line of defence against *A. fumigatus*. Inhaled *A. fumigatus* conidia can reach the alveoli where they germinate and subsequently invade blood vessels (Latge, 1999, 2001). At this stage of infection, *Aspergillus* germinates, growing small germ tubes (germinating conidia), followed by the generation of hyphal fragments. Immune cells of the peripheral blood, including polymorphonuclear neutrophils, dendritic cells and monocytes, attack *A. fumigatus* germlings and hyphae by phagocytosis, dragging or mere touching, thereby preventing further spread into other tissues (Behnsen *et al.*, 2007).

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Abbreviations: IA, invasive aspergillosis; IL, interleukin; SLR, signal log₂ ratio; TLR, Toll-like receptor; TNF, tumour necrosis factor.

After internalization of germlings and upon direct cellular contact with hyphae, different immune-relevant genes are activated.

Antigen-presenting cells, such as monocytes, recognize pathogens using receptors including Toll-like receptors (TLRs) (Blander & Medzhitov, 2004; Wang *et al.*, 2001), pentraxin-3 (PTX3) (Alles *et al.*, 1994; Vouret-Craviari *et al.*, 1997) and C-type lectins. PTX3 has been shown to bind microbial agents, including *A. fumigatus* conidia (Garlanda *et al.*, 2002), to facilitate phagocytosis. Extensive analysis of the role of inflammatory cytokines in cellular host defence, such as tumour necrosis factor alpha (TNF- α) (Frankenberger *et al.*, 1996; Roilides *et al.*, 1998), interleukin (IL)-6, IL-1 (Stordeur *et al.*, 2003) and IL-8 has been carried out in other studies. Chemokines, such as CCL2 (monocyte chemoattractant protein-1, MCP1) and CCL20 (MIP-3 α), have been shown to be significant contributors to chemotaxis (Allavena *et al.*, 1994; Loetscher *et al.*, 1994). The inflammatory response is accompanied by the regulation of a broad range of genes related to cell adhesion, apoptosis, transcription factors and heat-shock proteins.

In order to get a broader understanding of the genes involved in the host response to *A. fumigatus*, we analysed gene expression profiles in monocytes following incubation with *A. fumigatus* resting and germinating conidia, and ethanol-inactivated hyphae, in a time kinetic study. Analysis was performed by genome-wide expression profiling, as well as by real-time PCR and ELISA assays. In parallel, microscopic analysis of the phagocytosis of conidia was performed.

METHODS

Preparation of monocytes. Peripheral blood mononuclear cells (PBMCs) were separated from 500 ml heparinized blood from healthy donors by Ficoll-Hypaque density-gradient centrifugation (Biochrom). For analysis of phagocytosis, monocytes were isolated from PBMCs by positive labelling via CD14 microbeads using magnetic cell-sorting technology (Miltenyi). The purity of monocytes reached at least 95% by this positive selection procedure. In contrast, for genome-wide expression profiling, monocytes were isolated using a monocyte isolation kit (Miltenyi) with indirect magnetic labelling of non-monocytes (purity >75%). In this way, monocytes remained untouched, and stimulation by magnetic beads could be excluded. Cells were counted and resuspended in RPMI 1640 containing 25 mM HEPES buffer (Invitrogen) and 10% heat-inactivated fetal calf serum (Sigma-Aldrich).

Preparation of *A. fumigatus* isolates. *A. fumigatus* isolated from a patient suffering from IA (Institute of Medical Microbiology, Tübingen University Hospital) was cultivated for 5–7 days on Sabouraud 2% glucose agar (Merck) at room temperature. Conidia were harvested by washing the surface of the agar with PBS containing 0.01% Tween (Merck), and were washed with RPMI 1640 and counted in a Neubauer counting chamber. Conidia were then resuspended in RPMI 1640 and a defined number of monocytes was added. After 3 h of incubation, conidia were mostly still in a resting state, whereas after 6 h, conidia were swollen and germination

had started. After 9 h of incubation, the presence of germ tubes could be observed in all specimens.

A. fumigatus hyphae were prepared after culturing conidia for 18 h in 1 ml yeast nitrogen base (Merck), as reported by Denning (1996). Hyphae were inactivated in a 70% ethanol/PBS (Merck) solution for 24 h at 4 °C, washed twice with PBS and stored at –80 °C until further use. The efficacy of inactivation was controlled by cultivating hyphae on Sabouraud 2% glucose agar plates.

Phagocytosis assays. For phagocytosis assays, monocytes were labelled with a mouse anti-CD14 mAb, followed by goat anti-mouse Cy3-labelled antibody (both kindly provided by Christian Sinzger, Virology Institute, University of Tübingen) and incubated with germinating conidia at a ratio of 1:2 at 37 °C for 6 h. Two strategies were followed: (i) *A. fumigatus* conidia were harvested, washed and then stained with Fungi-Fluor dye (Polysciences) for 1 min, washed twice with RPMI 1640 and immediately incubated with the monocytes; or (ii) conidia were stained with Fungi-Fluor after 6 h of incubation with monocytes (using this approach, only non-phagocytosed conidia were stained). Phagocytosis rates were assessed by fluorescence microscopy.

Incubation assays and RNA isolation. For time-dependent gene expression analysis, monocytes from healthy donors ($n=3$) were incubated with different maturation forms of *A. fumigatus*: resting conidia, swollen and germinating conidia, and hyphae (for 3, 6 and 9 h). Briefly, 10^7 monocytes were incubated with 2×10^7 viable conidia or 10^6 hyphae at 37 °C and 5–10% CO₂.

RNA was isolated directly using an RNeasy mini kit and QiaShredder spin columns (Qiagen), according to the manufacturer's protocols. RNA concentration was measured by standard UV photometry (with a biophotometer; Eppendorf); an aliquot of RNA was taken for cDNA synthesis and the rest was frozen at –80 °C until further use.

Microarray analysis. For genome-wide expression analysis, microarray experiments were performed for every period of incubation and donor, using the HG-U133A array (Affymetrix). Briefly, double-stranded cDNA was synthesized from 5 μ g total RNA with a SuperScript Choice kit (Invitrogen). cRNA was prepared by *in vitro* transcription (Enzo Biochemical) and labelled with biotin. Fragmentation followed by incubation at 94 °C for 35 min in the presence of 40 mM Tris/acetate (pH 8.1), 100 mM KOAc and 30 mM MgOAc. Hybridization was performed with 15 μ g cRNA for 16 h at 45 °C. The arrays were then washed automatically, stained with streptavidin-phycoerythrin and scanned with a Genechip System confocal scanner (Agilent Technologies). Affymetrix Microarray Suite software (version 5.0), MicroDB, GCOS and the Data Mining Tool were used for analysis and evaluation of the arrays. Cluster analysis was performed using Genesis 1.3.0 (Institute for Bioinformatics), after determination of the mean values of the signals of the different incubation experiments. Functional analysis was performed using PathwayAssist software (Ariadne Genomics). For evaluation of up- or downregulated genes, as well as for cluster and pathway analysis, we set the baseline at signal log₂ ratios (SLRs) of <–1 and >1.

Real-time RT-PCR. In order to validate stimulation of monocytes prior to genome-wide expression profiling and to confirm differentially expressed genes detected by the arrays, a variety of real-time RT-PCR assays was established on a LightCycler (Roche). cDNA was synthesized using an AMV First-Strand cDNA synthesis kit (Roche). Amplification and quantification were performed using a standard LightCycler protocol (Roche). Primer and probes were supplied by TIB MOLBIOL. External cDNA standards (from 10^6 to 10^3 copies) were used for quantification. Normalization of samples was performed using the LightCycler h-ALAS housekeeping gene set (Roche).

ELISA. To confirm differentially regulated genes at the protein level, quantitative determination of cytokine and chemokine concentrations (IL-8, CCL2 and CCL20) in the supernatants was performed using Quantikine immunoassay kits (R&D Systems).

RESULTS

Phagocytosis of *A. fumigatus* conidia by human monocytes

Phagocytosis of resting conidia was visualized after 3 h of co-incubation. By staining conidia with fluorescent dye prior to (Fig. 1a, b, c) and after (Fig. 1d, e, f) phagocytosis, phagocytosed and non-phagocytosed conidia could be differentiated. By counting monocytes ($n=100$) in three independent experiments, the following mean values were obtained: 40 monocytes phagocytosed two conidia, 23 phagocytosed three, 16 phagocytosed one, 10 phagocytosed four or more and 11 phagocytosed none. Phagocytosed conidia were found to swell over time (Fig. 1d), and monocytes changed their shape accordingly.

Fig. 1. (a) Light microscopy of two monocytes, each of which has phagocytosed three conidia. (b, c) The same microscopic view as (a), but showing the monocytes labelled with mouse anti-CD14 mAb and secondary Cy3-labelled antibody (b), and the phagocytosed conidia (c), which were stained with Fungi-Fluor dye prior to phagocytosis. (d) Light microscopy of a monocyte containing one phagocytosed swollen conidia next to three unphagocytosed conidia. (e, f) The same microscopic view as (d), but showing the three unphagocytosed conidia stained with Fungi-Fluor after phagocytosis as an overlaid image (e) and as a fluorescence image (f). Magnification $\times 400$.

A. fumigatus differentially regulates expression of immune-relevant genes in monocytes

To obtain a broader understanding of the genes involved in *Aspergillus*-directed monocytic defence strategies, the gene regulation of monocytes was studied by genome-wide expression profiling after co-cultivation with different maturation states of *A. fumigatus*. A total of 602 genes was differentially regulated after 3 h of incubation with inactivated hyphae and 206 after incubation with resting conidia, taking into account the baseline cut-off set previously for the SLRs (<-1 and >1). At 6 and 9 h of incubation, the number of differentially regulated genes showed only slight differences between (swollen/germinating) conidia and hyphae: 418 and 399 genes, respectively, were differentially regulated after incubation with conidia, and 375 and 456 genes, respectively, were differentially regulated after incubation with hyphae (Table 1). Expression level varied depending on the incubation time and the morphological state of the fungus, with SLRs ranging from -3.7 to $+4.7$ for conidia, and from -4.1 to $+3.5$ for hyphae. Analysis revealed a wide range of differentially regulated immune-response genes including cytokines, chemokines and TLRs, as well as genes encoding signal-transduction and adhesion molecules.

Phagocytosis of conidia does not induce immediate cytokine gene expression

Although phagocytosis occurred during the first 3 h (Fig. 1), uptake of resting conidia did not induce expression of cytokines or chemokines as demonstrated by microarray (Table 2) and real-time PCR analysis (Fig. 2). In contrast, genes encoding adhesion and surface molecules were differentially regulated (Table 2). In contrast to resting conidia, hyphae induced the expression of genes encoding cytokines and chemokines such as TNF- α , IL-1 α , IL-1 β , IL-6, IL-10 and CCL7 by 3 h of incubation (SLRs of 1–1.8).

Comparison of differentially expressed immune-relevant genes induced by conidia and inactivated hyphae

Hyphae and conidia have different surface structures and therefore interact differently with immune cells (Rodland *et al.*, 2008). Accordingly, different gene expression profiles may result after pathogen–cell interaction and may result

Table 1. Number of differentially regulated genes in human monocytes after exposure to conidia and hyphae

No. of genes	Exposure to conidia			Exposure to hyphae		
	3 h	6 h	9 h	3 h	6 h	9 h
Upregulated	99	166	231	287	147	214
Downregulated	107	252	168	315	228	242
Total	206	418	399	602	375	456

Table 2. Selected differentially regulated genes after incubation with resting, swollen or germinating conidia

Values in parentheses represent the SLR.

Cytokine	Cytokine receptor	Chemokine	Chemokine receptor	TLR	Adhesion molecule	Other
Resting conidia (3 h)						
	IL-7R (−0.93, 0.76)				ITGAE (−0.73, 0.32)	CD86 (−0.95, 0.57)
	CSF1R (−0.73)				TFPI (−0.73)	STAT1 (−0.67)
					LGALS2 (2.05)	PTX3 (1.87)
					THBD (1.18)	MHCII α (0.68)
					SERPIN B9 (0.74)	MIR (0.7)
Swollen conidia (6 h)						
IL-8 (2.19)	TRAIL-R2 (−1.05)	CXCL5 (2.23)	CCR2 (−2.19, 1.51)	TLR1 (−1.22, 0.62)	F8A (−0.85)	NCF4 (−1.01)
TNFSF3 (1.11)	IFN γ R1 (−0.83)	CXCL3 (1.74)			PECAM1 (−2.09)	CASP8 (−1.43)
	IL-1RN (2.58)	CCL2 (1.48)			CAK β (−1.52)	MHCII β (−1.34, 0.66)
	IL-2R γ (1.03)				F3 (3.95)	MET (2.35)
	IL-3R α (1.0)				SERPIN B2 (1.16)	STAT3 (0.79)
					ALCAM (1.02)	
Germinating conidia (9 h)						
IL-8 (4.05)	CSF3R (−1.1)	CCL20 (4.3)	CCR2 (−2.03, 2.27)		CLECSF14 (−1.64)	SOCS3 (2.34)
IL-1 β (3.53)	TRAIL-R2 (−0.76)	CXCL5 (4.29)			LGALS2 (−2.17)	MET (2.21)
CSF1 (2.42)	IL-3R α (1.94)	CCL2 (2.33)			CD33 (−2.51)	MMP9 (1.99)
TGF- β 1 (1.18)	IL-2R γ (1.33)	CCL7 (1.39)			F3 (3.83)	MRC1 (1.94)
TNFSF15 (1.77)	CSF2R α (0.8)	CXCL1 (1.74)			SERPIN E1 (3.29)	C3 (1.39)
TNF- α (1.62)	TRAF1 (0.71)				uPA (1.62)	C1QR1 (1.35)
TNFSF3 (1.18)					ICAM-1 (1.59)	
					SERPIN B2 (1.33)	

ALCAM, Activated leukocyte cell adhesion molecule (CD166); C1QR1, complement component 1; C3, complement component 3; CAK β , cell adhesion kinase β ; CASP8, caspase 8; CCL5, regulated upon activation normal T expressed and secreted (RANTES); CCL7, monocyte chemotactic protein 3 (MCP3); CD33, sialic acid-specific lectin; CD86, B-lymphocyte activation antigen B7-2; CLECSF, C-type lectin; CSF1, colony-stimulating factor 1; CXCL1, melanoma growth stimulating activity (GRO1); CXCL3, oncogene (GRO3, MIP-2 β); CXCL5, neutrophil-activating protein 78; F3, coagulation factor III (CD142); F8A, coagulation factor VIII-associated protein; IFN γ R, interferon- γ receptor; ITGAE, integrin- α E (CD103); LGALS, lectin, galactoside-binding, soluble; MET, proto-oncogene (hepatocyte growth factor receptor); MHCII, major histocompatibility complex II; MIR, cellular modulator of immune recognition; MMP9, matrix metalloproteinase 9; MRC1, mannose receptor, C type 1; NCF4, neutrophil cytosolic factor 4; PECAM1, platelet/endothelial cell adhesion molecule (CD31); SERPIN, serine (or cysteine) proteinase inhibitor; SOCS3, STAT-induced STAT inhibitor 3; STAT, signal transducer and activator of transcription; TFPI, tissue factor pathway inhibitor; TGF, transforming growth factor; THBD, thrombomodulin (CD141); TNFRSF, tumor necrosis factor receptor superfamily; TRAF, tumour necrosis receptor-associated factor; TRAIL, TNF-related apoptosis-inducing ligand.

in different immune-response strategies. One of the notable differences was the downregulation of the gene encoding CCR2 (a 4-fold decrease; Table 2) after incubation with swollen and germinating conidia, but no differential expression after incubation with inactivated hyphae.

A. fumigatus germinating conidia and hyphae both stimulated cytokine and chemokine gene expression (Tables 2 and 3). Expression of the genes encoding IL-6, IL-8, IL-10 and IL-1 β was upregulated up to 280-fold. In addition, various chemokines such as CCL2, CCL7 and CCL20 and members of the GRO family were found to be upregulated. Germinating conidia selectively upregulated

expression of the genes encoding CSF1, TGF- β 1, TNF- α and CCL20.

Results were confirmed by real-time PCR for selected cytokine genes (Fig. 2). In addition, protein levels of CCL20, CCL2 and IL-8 were analysed and showed increased concentrations in culture supernatants after 6 and 9 h of incubation (Fig. 3).

Expression of the gene encoding IL-8 induced by activated conidia (germlings) and hyphae

Activated conidia may lead to differential monocytic gene expression, as conidia metabolism is augmented and

Fig. 2. Expression analysis of the genes encoding TNF- α , IL-1 β and IL-6 by real-time PCR. Three runs were performed for each gene and incubation period using identical mRNA, as in the microarray experiments.

cell-wall composition differs, compared with resting conidia and hyphae. Thus, we quantified expression of the gene encoding IL-8 after stimulation of monocytes with activated conidia and hyphae for 3, 6 and 9 h. Gene expression was maximal with both stimuli after 3 h of co-cultivation, decreasing after 6 and 9 h. However, gene expression varied widely: germlings induced a significantly higher level of IL-8-encoding gene expression than hyphae ($P=0.025$) compared with unstimulated cells (Fig. 4).

Expression of genes involved in pathogen recognition

No differential TLR2/TLR4-encoding gene regulation was detected by microarray analysis after 3, 6 and 9 h of co-cultivation with *A. fumigatus* conidia and hyphae. However, expression of the gene encoding PTX3, a lectin-like molecule, was notably enhanced in the early phase only. Real-time PCR assays showed upregulation of the gene by 40-fold after 1 h, 46-fold after 3 h, 15-fold after

6 h and 3-fold after 9 h when stimulated with conidia. Stimulation with hyphae revealed unchanged expression.

Differential regulation of uPA, uPAR and PAI, as well as ICAM-1, encoding genes after incubation with germinating conidia and hyphae

We could demonstrate upregulation of the genes encoding urokinase type plasminogen activator (uPA; SLR 1.7) and intercellular adhesion molecule-1 (ICAM-1; SLR 1.4) by gene array analyses. Due to their potential involvement in local inflammation and haemorrhage during IA, we further analysed the expression of genes encoding ICAM-1, uPA, the uPA receptor (uPAR) and the uPA receptor inhibitor (PAI) by real-time PCR. Interestingly, only conidia led to a notable upregulation of the ICAM-1-encoding gene (23-fold after 3 h, 3-fold after 6 h, 3-fold after 9 h), whereas hyphae did not induce expression.

In parallel, conidia were able to augment levels of the genes encoding uPA, uPAR and PAI after 3, 6 and 9 h of co-cultivation (Fig. 5). Hyphae led to reduced gene expression for uPA, unchanged gene expression for uPAR and increased gene expression for PAI (Fig. 5). All analyses were performed with monocytes isolated from three to six independent blood donors.

DISCUSSION

IA is a major cause of infection-related morbidity and mortality in immunocompromised patients. Conidia of *A. fumigatus* enter the lungs by inhalation where they germinate to form hyphae, which are capable of tissue invasion. Monocytes play a major part in innate immunity by initiating immune responses by phagocytosis, killing pathogens and producing a wide range of co-stimulatory molecules, and inflammatory cytokines and chemokines (Janeway & Medzhitov, 2002).

For a broader understanding of the defence strategies of peripheral monocytes against *A. fumigatus*, we compared data from interaction analyses of human monocytes with resting conidia (not present in physiopathological conditions), germlings and hyphae, by performing genome-wide expression profiling. Gene expression patterns were correlated with phagocytosis of conidia in a time-dependent manner.

The majority of monocytes were found to phagocytose up to three conidia within the first 3 h. The number of phagocytosed conidia remained unaltered after 6 h. However, conidia became swollen, and monocytes changed their shape accordingly. Conidia could partly escape phagocytosis, and non-phagocytosed conidia germinated to hyphae and overgrew the monocytes after 9 h.

Despite immediate phagocytosis of resting conidia, no differential regulation of genes encoding cytokines and chemokines could be observed. In contrast, hyphae

Table 3. Selected differentially regulated genes after incubation with inactivated hyphae

Values in parentheses represent the SLR. Abbreviations are defined below and in Table 2.

Cytokine	Cytokine receptor	Chemokine	Chemokine receptor	TLR	Adhesion molecule	Other
Hyphae 3 h						
IL-6 (1.77)	CSF2R α (−0.94)	CCL7 (1.21)	CXCR4 (−0.66)	TLR4 (0.72)	ALCAM (−1.34)	PTX3 (2.27)
IL-1 α (1.43)	TNFRSF6 (1.41)	CCL5 (0.82)	CCR5 (−1.07, 0.94)		LGALS2 (2.45)	NF κ B1 (0.54)
IL-10 (1.32)	TRAIL-R2 (0.96)				ADAM19 (2.04)	
IL-1 β (1.05)					ITGA2B (1.15)	
IL-8 (3.51)					uPA (0.78)	
					F13A1 (0.88)	
Hyphae 6 h						
TNFSF3 (1.95)	CRLF2 (1.62)	CXCL3 (1.73)		TLR7 (−2.71)	PECAM1 (−0.82)	C3 (1.04)
	TRAF1 (1.34)	CXCL1 (1.66)		TLR8 (−3.47)	F8A (−0.64)	MMP9 (1.26)
	IL-1R2 (0.78)	CXCL2 (0.85)			CLECSF2 (−1.32)	
					THBS1 (1.84)	
					SERPIN B2 (1.37)	
					uPAR (0.98)	
Hyphae 9 h						
IL-8 (1.53)	CSF3R (−1.21)	CCL2 (2.63)			CLECSF2 (−0.56)	CD244 (−1.53)
IL-1 α (2.7)	TRAIL-R2 (−0.84)	CXCL6 (2.56)			CLECSF14 (−2.21)	CD163 (−2.3)
TGF- α (1.88)	IL-1RAP (2.04)	CCL7 (2.12)			SERPIN E1 (2.4)	MMP9 (2.35)
	IL-7R (1.57)	CXCL1 (1.95)			THBD (2.29)	MET (1.68)
	IL-3R α (1.41)				uPA (1.81)	LAT (1.26)
	IL-2R γ (1.15)				ICAM-1 (1.22)	MAPK8 (1.21)
						C3 (0.96)

ADAM19, A disintegrin and metalloproteinase domain 19; CCR5, CD195 (agonists – MIP-1 α , MIP-1 β , RANTES); CD163, macrophage-associated antigen; CD244, natural killer cell receptor 2B4; CRLF, cytokine receptor-like factor; CSF1, colony-stimulating factor 1; CXCL2, oncogene (GRO2, MIP-2 α); CXCL6, granulocyte chemotactic protein 2 (GCP2); CXCR4, CD184 or fusin (agonist – SDF-1 α); F13A1, coagulation factor XIII, A1 polypeptide; ITGA2B, integrin- α 2b, platelet fibrinogen receptor, CD41B; LAT, linker for activation of T cells; MAPK, mitogen-activated protein kinase; NF κ B1, nuclear factor of kappa light polypeptide gene enhancer in B cells 1; THBS1, thrombospondin 1; TNFRSF, tumor necrosis factor receptor superfamily; TRAIL, TNF-related apoptosis-inducing ligand.

induced a strong differential expression of immune-relevant genes after 3 h of incubation. Thus, we concluded that resting conidia are only weak stimuli for monocytes, whereas germinating conidia and hyphae are able to induce a strong immune response. Similar results have been shown previously for the interaction of endothelial cells with *Pseudomonas aeruginosa* (Grassme *et al.*, 2003) where an inflammatory response could be detected in the later stages of phagocytosis only. Notably, preliminary experiments revealed that inactivation of *A. fumigatus* with 70 % ethanol did not significantly change the immune response of monocytes compared with living fungus.

Cortez *et al.* (2006) published their observations analysing the functional genomics of monocytes in response to *A. fumigatus* conidia. However, their data were limited to a maximum of 6 h of co-cultivation of conidia with monocytes, whereas our work was extended to 9 h (germinating conidia). As demonstrated, gene expression profiles changed significantly during the time-course experiments. Furthermore, we performed additional analysis of the interaction of monocytes with inactivated hyphae. Differential recognition of conidia and hyphae can skew downstream adaptive immune responses. This aspect

is of special interest, even if the total number of regulated genes did not significantly vary between 6 and 9 h (418 genes vs 399 genes).

Recently, Rodland *et al.* (2008) presented microarray data exploring gene expression in human monocytes infected with *A. fumigatus* conidia. However, again, their study was limited to an incubation time of 6 h, using a mixed population of conidial morphotypes; they reported that, after 5 h of incubation, more than 50 % of the conidia were swollen, with diameters >3.3 μ m.

Numerous genes involved in immune defence mechanisms were upregulated after 9 h of co-cultivation only. Among these were various chemokine genes, such as those encoding CCL7 and CCL20. CCL7 is involved in recruitment of macrophages to sites of inflammation. CCL20 shows activity towards neutrophils, monocytes and naive T lymphocytes and mobilizes intracellular calcium. In parallel, CCL20 was also found to be upregulated when *A. fumigatus* germinating conidia were co-cultivated with human dendritic cells (Mezger *et al.*, 2008), but not when cells were co-cultivated with *Streptococcus agalactiae* (C. Beyrich, personal communication).

Fig. 3. ELISA assays showing the concentrations (pg ml⁻¹) of IL-8, CCL2 and CCL20 in culture supernatants.

Expression of the genes encoding CCL7 and CCL20 is regulated by mediators of inflammation, such as TNF- α .

Fig. 4. Relative quantification of the expression of the gene encoding IL-8 by real-time PCR assay. The expression level in unstimulated monocytes after 3 h was given a value of 1. Expression analysis was from three independent donors and results are shown as means $\pm$ SD. Extr neg, Extraction negative control; Hy, hyphae; Ks, activated germinating conidia; MMX neg, PCR negative control; Mo, monocytes; Unst, unstimulated.

Fig. 5. Relative quantification of the expression of the genes encoding uPA, uPAR and PAI by real-time PCR assays. The expression level in unstimulated monocytes after 3 h was given a value of 1. Expression analysis was from three independent donors and results are shown as means $\pm$ SD. Extr neg, Extraction negative control; Hy, hyphae; Ks, activated germinating conidia; MMX neg, PCR negative control; Mo, monocytes; Unst, unstimulated.

We observed upregulation of genes encoding TNF- α and other proteins involved in the TNF- α pathway, such as TNFSF3 and TNFSF15. TNF- α is one of the key mediators involved in pro-inflammatory responses to bacteria and fungi. TNFSF15 is involved in endothelial-cell apoptosis via activation of the stress protein kinases SAPK/JNK and p38 MAPK, as well as caspase-3.

Interestingly, germinating conidia led to upregulation of expression of the CCL2-encoding gene (a chemokine that induces the recruitment of monocytes and neutrophils to infection sites) and, in parallel, to a downregulation of expression of the CCR2-encoding gene. Recent studies have described the significant role of CCR2 in clearance of *A. fumigatus* conidia from the airways of mice. CCR2^{-/-} mice showed reduced neutrophil recruitment into the airways, as well as an increase in inflammation and subepithelial

fibrosis (Blease *et al.*, 2000). Lundien *et al.* (2002) demonstrated that CCR2 expression is inhibited in bronchial epithelial cells when incubated with LPS, a method that may be used by bacteria to promote cell infection. Similarly, *A. fumigatus* germinating conidia were able to inhibit expression of the CCR2-encoding gene in monocytes, whereas hyphae obviously lost the ability to inhibit this expression.

In parallel to the data of Cortez *et al.* (2006), we revealed strong upregulation of IL-8, which is another chemokine that enhances the phagocytosis of *A. fumigatus* by polymorphonuclear neutrophils. Indeed, induction of cytokine production in monocytes appeared to be strongly dependent on fungal elements, on the cell population and on the incubation time. In a recent study, Simitsopoulou *et al.* (2007) analysed the interaction of the THP-1 monocytic cell line with hyphal fragments of *A. fumigatus* for 20 h. In contrast to our findings, using freshly isolated monocytes from peripheral blood and shorter co-incubation periods (3–9 h), they described significant downregulation of genes encoding CCL2 and CCL20, whereas other genes, such as that encoding CCL5, showed similar results (upregulation) in both studies. The study of Simitsopoulou *et al.* (2007) also showed that the antifungal drug voriconazole strongly induced the expression of immunomodulatory genes in THP-1 cells, compared with hyphal fragments alone.

Activation of immune defence mechanisms by *A. fumigatus* is dependent on so-called pattern-recognition receptors, such as the mannose receptor, integrins and C-type lectins, as well as TLRs and PTX3. Although the interaction of TLR2 and TLR4 with *A. fumigatus* has been reported previously in numerous studies, we found no significant change in expression of genes encoding TLR2 and TLR4 during the 3, 6 and 9 h incubation periods. Changes in the expression of genes encoding TLR2 and TLR4 most likely occur following longer exposure to the organism, as also suggested by Cortez *et al.* (2006) in their studies with conidia only.

PTX3 is a lectin-like molecule that plays an essential role in antigen recognition and defence against conidia of *A. fumigatus*, which are opsonized by PTX3 (Garlanda *et al.*, 2005). PTX3 production is induced by primary inflammatory signals and microbial moieties. Our data demonstrated that PTX3-encoding gene expression in monocytes was upregulated after 1 h of incubation by germinating conidia. Thus, PTX3 may act as a promoter of phagocytosis of conidia. These findings are in line with reports that describe the role of PTX3 in supporting phagocytosis of *A. fumigatus* conidia and in activating an antifungal cellular response (Garlanda *et al.*, 2002, 2005; Gaziano *et al.*, 2004).

Localized lung tissue damage and local thrombosis of the lung are major complications after allogeneic stem-cell transplantation. They occur in 25–55% of transplant patients and can account substantially for transplant-related mortality (Clark *et al.*, 1999; Weiner *et al.*, 1986). Our microarray data demonstrated that distinct mechan-

isms in monocytes, regulated by *A. fumigatus*, could be responsible for this observation. Augmented production of the chemokines CCL2, IL-8 and CXCL6 may contribute to morphological tissue damage in lungs and to local pulmonary haemorrhage. Furthermore, we found an increase in the expression of genes encoding uPA, uPAR and PAI after stimulation for 3 h with germinating conidia. uPA binds to its receptor (uPAR), resulting in the conversion of plasminogen to plasmin, which acts as a chemoattractant for monocytes (Syrovets *et al.*, 1997). In this context, plasmin induces expression of inflammatory cytokines and chemokines at sites of inflammation (Burysek *et al.*, 2002; Syrovets *et al.*, 2001; Weide *et al.*, 1996). Recently, it has also been demonstrated that plasminogen binds directly to *A. fumigatus* in a dose-dependent manner, influencing the pathogenesis of invasive fungal infections (Zaas *et al.*, 2008).

In contrast, hyphae led to decreased gene expression for uPA and increased gene expression for PAI. Impaired levels of uPA and augmented levels of PAI result in progression of thrombosis and fibrosis, and in suppression of fibrinolysis. In parallel, higher expression of the PTX3-encoding gene after incubation with *A. fumigatus* hyphae may also increase coagulation, as PTX3 increases tissue factor expression in activated monocytes, demonstrating an additional link between inflammation and thrombogenesis (Napoleone *et al.*, 2004). Additionally, we revealed increased expression levels of the gene encoding ICAM-1. ICAM-1 is a major adhesion molecule expressed on lymphocytes, and is also a critical accessory molecule for T cells. Increased levels of ICAM-1 result in oedema and haemorrhage. Furthermore, ICAM-1 leads to augmented infiltration of neutrophils and mast cells, which can be responsible for neutrophil-mediated local tissue injury (Westlin & Gimbrone, 1993; Yanaba *et al.*, 2003). Taking these observations together, we presume that, among other factors, reactions triggered by monocytes may partly explain local thrombosis and local tissue damage during IA.

In summary, gene expression profiling of monocytes co-cultivated with *A. fumigatus* resting and swollen conidia and germlings, as well as hyphae, in a kinetic profile extends our knowledge of the host–pathogen interaction of this clinically relevant mould. Our data indicate that monocytes, along with other immune-effector cells, such as neutrophils and macrophages, play a major role in innate immune defence by inducing an inflammatory response to *A. fumigatus* conidia and hyphae. Production of different chemokines, adhesion molecules and immune receptors may contribute to local thrombosis and lung tissue injury.

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