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Candida albicans Triggers Activation of Distinct Signaling Pathways to Establish a Proinflammatory Gene Expression Program in Primary Human Endothelial Cells

Verena Müller,* Dorothee Viemann,† Marc Schmidt,* Nicole Endres,* Stephan Ludwig,‡ Martin Leverkus,§ Johannes Roth,† and Matthias Goebeler2*†

Endothelial cells (EC) actively participate in the innate defense against microbial pathogens. Under unfavorable conditions, defense reactions can turn life threatening resulting in sepsis. We therefore studied the so far largely unknown EC reaction patterns to the fungal pathogen Candida albicans, which is a major cause of lethality in septic patients. Using oligonucleotide microarray analysis, we identified 56 genes that were transcriptionally up-regulated and 69 genes that were suppressed upon exposure of ECs to C. albicans. The most significantly up-regulated transcripts were found in gene ontology groups comprising the following categories: chemotaxis/migration; cell death and proliferation; signaling; transcriptional regulation; and cell-cell contacts/intercellular signaling. Further examination of candidate signaling cascades established a central role of the proinflammatory NF-κB pathway in the regulation of the Candida-modulated transcriptome of ECs. As a second major regulatory pathway we identified the stress-activated p38 MAPK pathway, which critically contributes to the regulation of selected Candida target genes such as CXCL8/IL-8. Depletion of MyD88 and IL-1R-associated kinase-1 by RNA interference demonstrates that Candida-induced NF-κB activation is mediated by pattern recognition receptor signaling. Additional experiments suggest that C. albicans-induced CXCL8/IL-8 expression is mediated by TLR3 rather than TLR2 and TLR4, which previously have been implicated with MyD88/IkB kinase-2/NF-κB activation by this fungus in other systems. Our study provides the first comprehensive analysis of endothelial gene responses to C. albicans and presents novel insights into the complex signaling patterns triggered by this important pathogen. The Journal of Immunology, 2007, 179: 8435–8445.

Invasive infections by pathogenic Candida species are a common cause of nosocomial bloodstream infections (1). Although dimorphic fungi like Candida albicans colonize distinct microanatomical regions such as mucous membranes as a commensal, occasionally they can also cause severe and often life-threatening disease, especially in patients with compromised immunity. Manifestations may then not only include oropharyngeal, vulvovaginal, or intertriginous candidiasis but also candidemia and invasive fungal infection when Candida gains access to the bloodstream. In such situations, Candida gets in close contact to the vascular endothelium that is strategically located at the border between blood and tissue compartments. In disseminated candidiasis, Candida adheres to and penetrates the endothelium resulting in cellular activation, injury, and eventually cell death (2–5). Increasing evidence points to a complex cooperation of different pattern recognition receptors (PRRs)1 in the host innate immune response to fungal pathogens that not only depend on species and morphotype but also on the route of infection (6, 7). Recently, the mechanism of C. albicans recognition by murine macrophages has been elucidated. In those cells, several sensory systems appear to work in parallel and include recognition of O-linked mannosyl residues on the outer layer of the C. albicans cell wall by TLR4, N-linked mannosyl residues by the mannose receptor, and β-glucans located at the inner layer of the C. albicans cell wall by the dectin-1/TLR2 receptor complex (8). However, the role of dectin-1 for C. albicans infection is still controversial (9, 10).

Interaction and response patterns of human endothelium to C. albicans have not been studied in detail yet. In this study, we used oligonucleotide microarray analysis to elucidate the endothelial gene expression program elicited by infection with Candida. We found that genes involved in the major categories of “chemotaxis/migration,” “cell death and proliferation,” “signaling,” “transcriptional regulation,” and “cell-cell contacts/intercellular signaling” were over-represented among the Candida-regulated transcripts, indicative for a broad inflammatory response induced by Candida in endothelial cells (ECs). Most striking in this respect was the strong induction of multiple neutrophil-attracting chemokines, which have not been identified in the context of Candida infection.

3 Abbreviations used in this paper: PRR, pattern recognition receptor; IKK, IκB kinase; EC, endothelial cell; RNAi, RNA interference; shRNA, small hairpin RNA; siRNA, small interfering RNA; IRAK, IL-1R-associated kinase; poly(I:C), polyinosinic-polycytidylic acid; MOI, multiplicity of infection.
before. We furthermore demonstrate that the well-known proinflammatory IkB kinase (IKK)/IkB/NF-κB signaling pathway plays a pivotal role in establishing the inflammatory response toward *Candida* and that activation of the p38 MAPK cascade is crucial for *Candida*-induced expression of selected genes in ECs such as CXCL8/IL-8. Finally, we provide evidence that in the endothelial system the proinflammatory gene response to *Candida* is a receptor-mediated event depending on the adapter molecule MyD88 and on IL-1R-associated kinase-1 (IRAK1) without apparent involvement of the common microbial PRRs TLR2 and TLR4. Rather, RNA interference (RNAi) experiments suggest TLR3 as mediator of *Candida*-induced proinflammatory gene expression in ECs.

### Materials and Methods

#### Cytokines and reagents

Human recombinant TNF-α, IL-1β, and IL-1R antagonist were obtained from R&D Systems. The pharmacological p38 inhibitor SB203580 was purchased from Calbiochem, the TLR2/TLR6 agonist macrophage-activating lipopeptide-2 (MALP-2) from Alexis, and the TLR agonists Pam3CSK4, polyinosinic-polycytidylic acid (polyIC), and fibroblast-stimulating lipopeptide-1 (FSL-1) from InvivoGen, respectively. All other agents were obtained from Sigma-Aldrich, unless specified otherwise. Soluble TNF receptor fusion protein (TNFR2-Fc) was obtained from Wyeth Pharmaceuticals.

#### Culture of ECs and *C. albicans*

Primary HUVEC were purchased from Clonetics (via Cambrex Biosciences). Cells were used between passages 3 and 5 in all experiments and cultured in a 1:2 mixture of endothelial growth medium (Clonetics) and M199 medium (PAA) supplemented with 6.7% FBS, gentamicin, amphotericin, and Liquemin N5000 (Roche). Twenty-four hours before addition of C. albicans, HUVEC were washed and placed in antibiotic-free medium.

#### Plasmids

A 6xNF-κB.Luc construct containing six copies of a NF-κB-binding motif cloned upstream of a minimal promoter driving a luciferase reporter gene was as well as an ubiquitin-dependent Renilla luciferase construct were obtained from B. Baumann (University of Ulm, Ulm, Germany). The pCFG5-IEGZ retroviral vector has been described earlier (11). A dominant negative mutant of human MyD88 (aa 152–296) was provided by Tularik Inc. (South San Francisco, CA) and cloned into the pCFG5-IEGZ retroviral backbone using 250 ng/reaction HindIII and Sall restriction enzymes. For RNAi studies, HUVEC were infected with retroviral supernatants. The pharmacological p38 inhibitor SB240440 (SB202190) was obtained from Wyeth Pharmaceuticals.

#### Quantitative real-time RT-PCR

cDNA was synthesized from 4 μg of total RNA using SuperScript II RNase H reverse transcriptase (Invitrogen Life Technologies). Specific primers for each gene (sequences available upon request) were designed using Primer Express software package (Applied Biosystems) and obtained from MWG Biotech. Quantitative RTPCR was performed using the Quantitect SYBR Green PCR kit (Qiagen) as described (13) and data acquired with the ABI PRISM 7900 (Applied Biosystems). Gene expression was normalized to the endogenous housekeeping control gene GAPDH, and relative expression of respective genes was calculated using the comparative threshold cycle method as described (14).

#### Immunoprecipitation, immune complex kinase assay, and Western blotting

ECs were lysed in Triton x-114 buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 50 mM sodium β-glycerophosphate, 20 mM sodium orthovanadate, 0.5 mM sodium fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 mM benzamidine). Immune complex kinase assays for p38 and IKK2 were essentially performed as described (15, 16) using recombinant ATP-2 or GST-IκBα as substrates for p38 or IKK2, respectively. Western blot analysis using polyclonal antiserum against p38 (C-20) or IKK2 (H-470; Santa Cruz Biotechnology) was performed to confirm equal loading of p38 and IKK2 proteins.

For analysis of IκBa expression and degradation cells were lysed in E1A lysis buffer (150 mM sodium chloride, 50 mM HEPES (pH 7.5), 5 mM EDTA and 1% Nonidet P-40) freshly supplemented with 20 mM sodium β-glycerophosphate, 0.5 mM sodium orthovanadate, and a commercial protease inhibitor cocktail (complete; Roche Molecular Biochemicals). Western blots were then performed as previously described (17) using polyclonal rabbit antiserum against IκBα (C-21, sc-71; Santa Cruz Biotechnology). IkBa and IκBa levels were visualized by ECL.

#### ELISA

Supernatants of HUVEC were collected after exposure to *C. albicans* or cytokines, centrifuged at 2600 × g to remove cellular debris and analyzed for synthesis of CXCL8/IL-8 using a commercial ELISA system (BD Biosciences) according to the manufacturer’s instructions. For CCL20/MIP-3α, HUVEC were lysed in Triton x-114 buffer, and protein levels of CCL20/MIP-3α in the lysates were determined using an ELISA detection kit for CCL20/MIP-3α (R&D Systems).

### Retroviral infections and retrovirus-mediated expression of shRNA

For overexpression of dominant negative versions of IKK2, retroviral infections of HUVEC were essentially performed as described (11). Briefly, the amphotrophic producer cell line dNXX was stably transfected with the indicated retroviral vectors by calcium phosphate precipitation or Lipo-Transfectamine 2000 (Invitrogen Life Technologies) and supernatants from these cells were used to infect HUVEC in two consecutive rounds. After selection for zeocin resistance conferred by a gene expressed from the retroviral backbone using 250 μg/ml zeocin, stably transduced HUVEC...
were incubated in conventional endothelial growth medium without antibiotics and antimycotics. Cells were subsequently exposed to *C. albicans* and processed for further analysis. Retroviral infection efficiency of HUVEC was routinely monitored by flow cytometric determination of GFP positivity due to marker gene expression from the retroviral constructs and consistently reached values of >85%.

For RNAi studies, HUVEC infected with the pRS retrovirus were selected by treatment with 1 µg/ml puromycin for at least 48 h. Cells were further incubated for additional 18 h in puromycin-free medium before stimulation in the respective experiments. Knockdown efficiencies in the selected cell population were routinely controlled by Western blot analysis.

**Transient transfection of HUVEC and reporter gene assays**

ECs were cultured to 50–60% confluence before transient transfection according to a DEAE-dextran protocol (18). Briefly, cells were incubated with 4 µg/2 × 10^6 cells 6xNF-kB.luc reporter construct, 133 ng/2 µg/mL HEPES/PBS for 30 min at 37°C. Cells were then incubated with 0.15 mM chloroquine in M199 medium for 3 h and, after removal of medium, exposed to 10% DMSO in M199 medium for another 2.5 min. After 16 h, cells were transferred to 96-well plates and stimulated with *C. albicans* or other reagents as described. Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). Values were each normalized to the luminescence generated by the cotenused Renilla luciferase control reporter.

HUVEC cultured on 6-well plates were transfected with validated small interfering RNA (siRNA) against human MyD88 (catalog number SI00300909), TLR3 (SI02655156), or TLR4 (SI00151004), all from Qiagen or a scrambled siRNA at a final concentration of 200 nmol/L using Oligofectamine (Invitrogen Life Technologies) according to the manufacturer’s protocol. Cells were exposed to *C. albicans* or other stimuli 48 h later, and chemokine synthesis determined by Western blot or ELISA analyses.

**Results**

*C. albicans* modulates expression of multiple genes in primary ECs

ECs do not only represent a physical barrier between different tissue compartments but are also capable of regulating defense mechanisms against a multitude of microbial pathogens. To investigate the response of human primary ECs to *C. albicans*, we performed gene profiling using Affymetrix U133A Gene Chip microarrays, which cover at least 18,400 transcripts, including 14,500 well-characterized human genes. To this end, *C. albicans* blastospores (strain SC5314) were cocultured with HUVEC for 5 h and total RNA from four independent experiments was individually processed for DNA microarray hybridization. A relatively early time point was chosen to minimize indirect autocrine or paracrine effects. In initial experiments we excluded a potential contamination of *C. albicans* preparations by LPS that might confound endothelial gene expression: neither *Limulus* amebocyte assays (data not shown) nor experiments with the LPS inhibitor polymyxin B sulfate provided evidence for a potential contamination of our *Candida* preparations with LPS (data not shown). To increase the reliability of our data sets, we further applied rigorous inclusion criteria in our microarray experiments and only considered transcripts as significantly regulated that showed at least a 2.5-fold alteration in gene expression levels. According to these criteria, we overall identified 56 genes that were up-regulated, whereas 69 were down-regulated upon exposure of ECs to *C. albicans*. Genes induced by the yeast could be assigned to the following GO categories: chemotaxis/migration, cell death and proliferation, signaling, transcriptional regulation, cell-cell contacts/intercellular signaling, and inflammatory response/cytokines (Fig. 1). Most of the genes have not been described as *Candida*-regulated genes before. Remarkably, a considerable number of up-regulated genes were chemokines that primarily attract neutrophils, suggesting that ECs contribute to the recruitment of leukocytes to sites of the infectious challenge. Among the down-regulated endothelial genes many belonged to the GO group “integral to plasma membrane” (data not shown). A subset of genes suppressed by *Candida* is related to glutamate-gated channels and associated signaling pathways (i.e., GRIK2, GRID2, GAD2). A complete list of *C. albicans*-regulated genes has been deposited in the National Center for Biotechnology Information GEO under accession number GSE7355 (and is available at http://www.ncbi.nlm.nih.gov/projects/geo/). To verify the gene expression data obtained in the microarray analysis by an independent method, selected genes were studied by quantitative

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**FIGURE 1.** Assignment of *Candida*-regulated genes to functional categories. To identify functional categories of genes statistically over-represented among the *Candida*-regulated transcripts, Gene Ontology (GO) annotations were assigned to every probe set spotted on the Affymetrix U133A Gene Chip. The representation of single GO annotations within the group of up-regulated genes in relation to the totally represented GO annotations on the chip was calculated using Fisher’s exact test. The diagram displays all statistically relevant GO groups among the up-regulated genes. p ≤ 0.01. Single GO groups are further assigned to the indicated categories (distinguished by color) with the most over-represented GO groups represented by the longest histogram corresponding to the lowest calculated p value.
real time RT-PCR. Highlighting the reliability of our stringent statistical analysis, up-regulation of these exemplary genes that included chemokines ICAM-1 and VCAM-1 could clearly be confirmed in all cases (Fig. 2A). Our data thus demonstrate that ECs exhibit an impressively strong gene expression response to C. albicans.

For additional experiments that aimed to identify intracellular signal transduction pathways influenced by C. albicans, we focused on CCL20/MIP-3α and CXCL8/IL-8 as read-out molecules. First, we studied protein expression of these chemokines in response to increasing MOI of Candida blastospores by ELISA and Western blot. Robust induction of CXCL8/IL-8 and CCL20/MIP-3α was achieved at an MOI ≥ 0.5. In line with the mRNA data, a clear induction of chemokines was also detected at the protein level within 8 h upon infection with MOI ≥ 0.5 of C. albicans (Fig. 2, B and C). Thus, an MOI of 1 at which a solid induction was found in all assays was chosen for subsequent experiments. Only viable C. albicans blastospores were capable of inducing chemokine expression, whereas heat-inactivated yeast did not elicit such a response even at MOI values of up to 4 (Fig. 2D).

C. albicans activates the IKK2/IκBα/NF-κB signaling pathway

Many of the detected Candida-regulated genes are known targets of the proinflammatory transcription factor NF-κB, which is activated via the IKK signalosome (12, 19, 20). To explore whether C. albicans induces activation of the NF-κB signaling pathway, we performed immune complex kinase assays to study the activity of the IκBα-phosphorylating kinase IKK2. In time course experiments, we observed an induction of IκBα phosphorylation within 2 to 4 h after infection with C. albicans (Fig. 3A, left). Consistent with our previous data (21), TNF-α stimulation resulted in a considerably faster and transient IκBα phosphorylation with maximal levels at 20 min (Fig. 3A, right). The kinetics of C. albicans-induced IKK2 activity was then compared with the kinetics of IκBα degradation for both stimuli. In each case, the timing of IκBα degradation strictly correlated with the activity of IKK2 (data not shown). Because both stimuli triggered IκBα degradation, we next determined the transcriptional activity using a luciferase reporter gene construct (Fig. 3B).

We detected a 3- to 4-fold induction of NF-κB-dependent transcriptional activity with C. albicans, whereas stimulation with TNF-α resulted in a 15- to 20-fold induction of the reporter gene (Fig. 3B). Because the kinetics of C. albicans-induced NF-κB activation appeared to be delayed as compared with TNF-α we wondered whether C. albicans might activate ECs indirectly via autocrine or paracrine action of IL-1β or TNF-α, two cytokines that are strong activators of NF-κB. However, neither the presence of IL-1R antagonist nor addition of a soluble TNFR2 fusion protein (TNFR2-Fc) could prevent Candida-induced CXCL8/IL-8 induction, whereas effects of IL-1β and TNF-α were completely blocked upon exposure of cells to their respective antagonists (Fig. 3C). Furthermore, supernatants of Candida-exposed ECs (i.e., conditioned medium) were not capable of inducing CXCL8/IL-8 expression, excluding the involvement of other soluble factors (Fig. 3D). Altogether, these data indicate that C. albicans is most likely a direct inducer of NF-κB activation in primary human ECs, albeit its stimulation capacity is weaker and delayed when compared with TNF-α.

C. albicans-mediated chemokine expression critically depends on the NF-κB signaling pathway

To investigate whether NF-κB is critical for C. albicans-induced chemokine expression, we next studied whether stable expression of a dominant negative mutant of IKK2 (IKK2KD) in ECs can abrogate induction of selected chemokines after exposure to C. albicans. Indeed, up-regulation of various transcripts including...
dependent transcription. HUVEC were transiently transfected with a NF-
previously exposed to
induce CXCL8/IL-8. Conditioned medium was collected from HUVEC
supernatants were determined. Induced levels obtained after incu-
filtration added to independent EC cultures for 8 h. As positive control,
stimulation
C. albicans
was confirmed by Western blot using an Ab against IKK2 (\textcopyright). One of
at a MOI of 1 (\textcopyright), TNF-
interactions with the indicated stimuli in nonsupplemented culture medium were
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HuVEC were preincubated with IL-1R antagonist (IL-1RA, 200 ng/ml), a
left
C. albicans
right
C. albicans
his expression as determined by flow cytometric analysis of GFP expression
for 8 h and subsequently analyzed for
expression with similar
indicates that
C. albicans-induced chemokine induction, indicating that C. albicans-induced expression of both chemokines critically requires activation of the IKK2/NF-\kappa B pathway.
C. albicans activates the p38 MAPK pathway
Efficient induction of CXCL8/IL-8 and other chemokines may additionally require activation of the p38 MAPK (12,22). To determine whether C. albicans also activates the p38 pathway, we used immune complex kinase assays using ATF-2 as a substrate. Fig. 5A indicates that C. albicans activated endothelial p38 with similar kinetics as observed for IKK2 (after 2–4 h), whereas TNF-\alpha induced a transient p38 activation with a maximum at 20 min followed by a steady decline of activity. To confirm the functional relevance of p38 for C. albicans-induced chemokine induction,
C. albicans-induced endothelial NF-κB-dependent gene expression requires IRAK1 and MyD88 but occurs independently of TLR2 and TLR4.

Having shown the importance of NF-κB- and p38-dependent signal transduction pathways for C. albicans-induced gene expression, we next focused on critical upstream signaling components. Because TLR2 and TLR4 have been implicated with pattern recognition of C. albicans in the murine system (8), we first studied the role of both receptors for Candida-dependent signaling in our coculture model. LPS, a known TLR4 agonist, strongly induced CXCL8/IL-8 synthesis (Fig. 6A, left) and NF-κB-driven transcriptional activity in HUVEC (data not shown). To our surprise the TLR2 agonist Pam3CSK4 neither stimulated endothelial CXCL8/IL-8 production (Fig. 6A) nor 6xNF-κB-dependent luciferase activity. Failure of Pam3CSK4 activation of HUVEC was not due to the agonist itself because the same preparation of Pam3CSK4 strongly induced CXCL8/IL-8 expression in the TLR2-positive monocytic cell line THP-1 (Fig. 6A, right). Moreover, agonists of TLR2/TLR6 (MALP-2 and FSL-1) did not elicit chemokine expression in ECs while they strongly up-regulated CXCL8/IL-8 in THP-1 cells. These observations suggest that, at least under our culture conditions, HUVEC do not contain functional TLR2 receptors. To further assess the role of TLR2 and TLR4 for Candida-induced signaling, we performed experiments with HEK293 cells that do not express significant levels of endogenous TLR2 and TLR4 (data not shown). Alternatively, transgenic HEK293 cell lines stably expressing either TLR1/TLR2 or TLR4/CD14-MD2 were used. Although parental HEK293 cells did not respond to stimulation with Pam3CSK4 or LPS (Fig. 6, B and C, left), HEK293-hTLR1/TLR2 cells produced high levels of CXCL8/IL-8 and significant 6xNF-κB-dependent luciferase activity upon stimulation with Pam3CSK4 but not LPS (Fig. 6, B and C, middle). Conversely, HEK293-hTLR4/CD14-MD2 showed increased CXCL8/IL-8 synthesis and κB-driven luciferase activity in response to LPS but not to Pam3CSK4 (Fig. 6, B and C, right). Importantly, stimulation of none of the diverse HEK293 cell lines with C. albicans blastospores resulted in CXCL8/IL-8 production or increased 6xNF-κB-dependent luciferase activity. In line with these observations, knockdown of endogenous TLR4 in HUVEC by RNAi using validated duplex siRNA did not block C. albicans-induced expression of CXCL8/IL-8, whereas the response induced by the TLR4 agonist LPS was considerably inhibited (Fig. 6D). We therefore have no evidence that TLR2 or TLR4 are critically involved in C. albicans-induced NF-κB-dependent gene expression in HUVEC or HEK293 cells. Studying expression of potential receptors for C. albicans in ECs, we also failed to detect significant mRNA levels of TLR2 and TLR6 and other potential receptors for yeast recognition such as dectin-1 and mannose receptor-1 under our culture conditions (data not shown).

Signaling via many PRRs requires recruitment of the adaptor protein MyD88 and subsequent activation of IRAK1. MyD88 and IRAK1 are thus functionally located at the bottleneck of PRR-dependent signaling. We therefore decided to knockdown endothelial IRAK1 and MyD88 by RNAi. The shRNA expression against IRAK1 resulted in a complete depletion of IRAK1 protein in HUVEC (Fig. 7A). Expectedly, ablation of IRAK1 expression had no significant effect on TNF-α-mediated expression of CXCL8/IL-8, whereas IL-1β-induced expression of the chemokine was largely blocked. Likewise, Candida-induced synthesis of CXCL8/IL-8 and CCL20/MIP-3α was almost completely abrogated in HUVEC stably expressing shRNA against IRAK1 (Fig. 7, A and B). Similar results were obtained with a dominant negative mutant of IRAK1 (IRAKKΔ53-54) (23) that was stably expressed in
HUVEC (data not shown). Similar to IRAK1 depletion, transfection of ECs with validated siRNA against human MyD88 efficiently blocked C. albicans- and IL-1β-induced expression of CXCL8/IL-8 and CCL20/MIP-3α (Fig. 7, C and D). Consistently, expression of a dominant negative mutant of MyD88 largely inhibited C. albicans-induced production of CXCL8/IL-8 (Fig. 7E).
These observations unequivocally demonstrate that *C. albicans* induces NF-κB-dependent gene expression via a MyD88/IRAK1-dependent signaling pathway. Because our data thus far suggested that neither TLR2 nor TLR4 or autocrine factors (compare Fig. 3D) can sufficiently account for *Candida*-induced NF-κB activation, we considered the involvement of other TLRs. Recently, Morris et al. (24) provided evidence that TLR3 can trigger proinflammatory gene expression in ECs in response to poly(I:C), an established TLR3 agonist. In search for other candidate TLRs, we thus tested whether functional TLR3 is also present in HUVEC under our culture conditions. Indeed, treatment of HUVEC with poly(I:C) resulted in a concentration-dependent expression of CXCL8/IL-8, demonstrating the presence of functional TLR3 in our cells (Fig. 8A). As controversial data have been published concerning the dependency of TLR3 signaling on MyD88 (25), we next analyzed whether siRNA mediated depletion of MyD88 can affect poly(I:C)-induced CXCL8/IL-8 expression. Fig. 8B illustrates that MyD88 depletion reduces poly(I:C)-mediated CXCL8/IL-8 induction, suggesting that this response is to a considerable degree dependent on MyD88. Finally, to prove that TLR3 is involved in *Candida*-induced proinflammatory responses, we depleted TLR3 by siRNA. Although TLR3 siRNA transfection had no effect on TNF-α-induced CXCL8/IL-8 expression, both *Candida*- and poly(I:C)-mediated CXCL8/IL-8 induction was clearly reduced (Fig. 8C). Taken together, these data suggest that the *Candida*-induced proinflammatory response is at least partially dependent on TLR3.

**Discussion**

The course of *Candida* infections is determined by both pathogen-specific and host-dependent factors (26, 27). Prominent target cells of *Candida* infection are ECs, which play a pivotal role in the early immune response during candidemia and invasive fungal infection as well as during sepsis. In such situations, *Candida* adheres to and invades the endothelium resulting in cellular activation and injury (2–4, 6). Using microarray analysis of human primary ECs, we identified 56 genes that were significantly up-regulated and 69 endothelial genes that were significantly suppressed upon coculture of HUVEC with *C. albicans* blastospores. By bioinformatical approaches we could identify those groups of genes that were significantly over-represented among the regulated genes. Most prominent were GO groups that cover chemotaxis and migration (Fig. 1). Overall, we identified six chemokines that are up-regulated by *C. albicans*. Remarkably, most of those represent factors that usually recruit neutrophils to sites of pathogen challenge. Because neutrophils play a pivotal role in the resolution of fungal infections (28) with neutropenia being a major indicator of poor prognosis, these findings suggest that ECs are a fully integrated player in an orchestrated defense network against fungal pathogens. Endothelial chemokines elicited by *C. albicans* include CXCL8/IL-8, CXCL1/GRαa, CXCL2/GRβ, CXCL3/GrαY as well as CXCL5/ENA78 and CXCL6/GCP-2, which were detected by quantitative RT-PCR. Induction of those chemokines was a direct effect of *Candida* exposure and not a consequence of autocrine IL-1 or TNF-α secretion because addition of soluble TNFR (TNFR2-Fc) or IL-1R antagonist to the coculture system failed to block their expression. Furthermore, conditioned medium was unable to elicit a CXCL8/IL-8 response excluding the involvement of other soluble factors. Thus, ECs most likely initiate their inflammatory response to *Candida* independently of host cell- or fungus-derived soluble factors.

CXCL20/MIP-3α, another previously unknown *Candida*-regulated endothelial chemokine, belongs to a subgroup of chemokines with defensin-like antimicrobial activity (29) and is a potent chemoattractant for dendritic cell precursors. CCL20/MIP-3α thus has the properties to act as a danger signal, thereby linking the innate to the adaptive immune system (30), which both determine the course of *Candida* infection in hosts (31). Besides cytokines a number of cell surface receptors were likewise induced upon *C. albicans* infection. These include tissue factor, the initiator of the extrinsic coagulation cascade, adhesion molecules such as ICAM-1 and VCAM-1 that are required for leukocyte recruitment, CD24, a receptor for P-selectin (32), and the chemokine receptor CXCR7 (chemokine orphan receptor 1). Furthermore, CD74, an
accessory signaling receptor resulting in NF-κB activation and cell survival (33), and the activation-associated receptor CD69 are up-regulated upon infection with C. albicans. Other over-represented functional groups of Candida-induced genes can be classified into the main categories cell death and proliferation, signaling, transcriptional regulation, and cell-cell contacts and intracellular signaling. A significant fraction of genes annotated to “transcriptional regulation” were zinc finger proteins that included Krüppel-like transcription factor KLF4, ZFP36 (also known as tristetraprolin), ZNF151, and Lin28. Although ZFP36 is up-regulated during inflammatory responses, it fulfills anti-inflammatory tasks required for the resolution of inflammatory reactions via mRNA destabilization of cytokines including TNF-α (34). Among the up-regulated genes many have been reported to act as negative regulators of cell proliferation, indicating that EC proliferation ceases after Candida infection.

Systematic analysis of the Candida-regulated gene repertoire revealed that many genes are under control of the transcription factor NF-κB, which orchestrates proinflammatory as well as antiapoptotic responses to various stimuli such as IL-1, TNF-α, or pathogens in multiple cell types (35). In this study, we demonstrate for the first time that Candida activates IKK2 resulting in phosphorylation and subsequent degradation of IκBα as well as NF-κB-dependent transcription. However, in sharp contrast to the prototypical activator of endothelial chemokine expression TNF-α, the kinetics of IKK2 activation by Candida is markedly delayed, which is in line with reports on other stress-like endothelial stimuli such as PMA, LPS, or nickel chloride (21, 36, 37). Delayed and persistent activation imply a different mode of activation. Therefore, it was mandatory to further dissect the intracellular requirements of Candida-induced gene expression. Upon stable expression of a dominant negative mutant of IKK2, which prevents activation of NF-κB, expression of various Candida target genes was blocked (see Fig. 4). This result clearly demonstrates NF-κB activation as a critical requirement for the expression of many Candida-regulated genes.

IKK2/NF-κB-dependent gene expression is often modulated by coregulatory signals generated by kinases of the MAPK family (38). In particular the p38 MAPK pathway is known to be involved in the regulation of several proinflammatory mediators in ECs such as CXCL8/IL-8 and CCL2/MCP-1, which exert functional roles in host defense responses (17, 18, 39). Albeit the mechanism of p38-dependent gene regulation is not completely understood, increasing evidence suggests an involvement of this pathway in specific pathophysiological events that result in stabilization or destabilization of mRNA (22, 40). Furthermore, p38 may modulate gene expression at the level of transactivation by interacting with components of the enhancerome, such as the transcriptional coactivator CBP/p300 (21). Highlighting the regulatory complexity, mitogen- and stress-activated protein kinase-1 (MSK1), which acts downstream of p38, has been identified to phosphorylate the NF-κB/Rel family member p65 as well as components of the chromatin environment, thereby interfering with NF-κB-dependent gene transcription (41, 42). Our data demonstrate that p38, like NF-κB, is activated in a delayed kinetic upon exposure to C. albicans. This finding is in agreement with a recent report studying HeLa cells (43). The kinetics also matches findings from previous studies using LPS (39) and nickel compounds (21) as endothelial activators. We additionally identified differences regarding the requirement of a coordinated activation of p38 and NF-κB signaling pathways; while CCL20/MIP-3α was largely independent of p38, CXCL8/IL-8 expression was clearly suppressed by a pharmacological inhibitor of p38. These data indicate that activation of NF-κB and p38 is critical for IL-8/CXCL8 induction, whereas p38 is dispensable for Candida-induced CCL20/MIP-3α expression. This result supports our previous observations (using other proinflammatory stimuli for ECs) that some NF-κB target genes are co-regulated by p38, whereas others obviously do not depend on p38 activation (12).

The ability of Candida to grow in different forms as a parasite or a commensal in vivo might have resulted in a complex repertoire of antifungal host response patterns. In systemic candidiasis, primarily cells of the innate immune system are required for an effective host defense. They need to reliably recognize microbial pathogens, which is ensured by evolutionarily ancient PRRs including TLRs, C-type lectin receptors, NOD proteins, and others (9, 10, 44, 45). Upon pathogen recognition, cells appear to activate intracellular signal transduction pathways, which ultimately results in gene expression and cellular activation. Recognition of C. albicans by mouse macrophages could recently be attributed to sensing of O-linked mannosyl residues of the outer layer of the C. albicans cell wall by TLR4, of N-linked mannosyl residues by the mannose receptor, and of β-glucans localized in the inner layer of the yeast cell wall by the dectin-1/TLR2 receptor complex (8, 9). Our data demonstrate that at least in primary human ECs alternative sensory mechanisms must exist that mediate responses to Candida. While ECs express substantial levels of TLR4 (46), we failed to detect significant amounts of functional TLR2 in HUVEC under our culture conditions (data not shown). In line with this result, ECs responded to neither the TLR1/TLR2 agonist Pam3CSK4, nor to the TLR2/TLR6 ligands MALP-2 and FSL-1 at concentrations that maximally induced CXCL8/IL-8 expression in TLR2-positive THP-1 cells. Candida-infection was further unable to trigger NF-κB activation and CXCL8/IL-8 expression in either parental HEK293 cells lacking TLR4 or HEK293 cells stably expressing TLR4 (together with MD-2/CD14); however, the latter cells were responsive to the TLR4 agonist LPS. It therefore seems unlikely that TLR4 is critical for signal perception of the yeast even more because siRNA against TLR4 failed to abolish Candida-induced CXCL8/IL-8 expression in ECs. According to our expression analysis (data not shown) and in line with an earlier report (47), dectin-1, the myeloid cell-expressed major receptor for fungal β-glucan, is not expressed by ECs thus excluding a role in Candida-induced endothelial signaling. Furthermore, the mannose receptor, another C-type lectin receptor, is absent in HUVEC (48). Thus, neither C-type lectin receptors nor TLR2 and TLR4 appear to be responsible for C. albicans-induced gene expression in our EC model.

Remarkably, RNAi-mediated depletion of the adapter molecule MyD88 as well as of IRAK1, a kinase downstream of TLRs, abolished C. albicans-induced expression of NF-κB target genes such as CXCL8/IL-8. This observation clearly indicates that endothelial activation by Candida is a receptor-mediated process. Dependency on MyD88 and IRAK1 suggests sensing of C. albicans by a TLR. Our siRNA data indicate that most likely TLR3 represents this receptor. TLR3 was initially reported as intracellular receptor primarily implicated with the perception of viral dsRNA and endogenous RNA species released upon cell damage (49–51). These data are compatible with a scenario in which viable yeasts enter a cell and cause a certain degree of EC injury as suggested by Filler et al. (5). Such damaging events may be associated with development of putatively endogenous dsRNA species that successively activate endothelial defense mechanisms via TLR3. Although the exact mechanism of Candida-induced TLR3 activation remains to be elucidated, a model of Candida-induced cell damage might also account for the observed delayed kinetics of Candida-induced signaling and provide a reasonable explanation why only viable C. albicans is capable of inducing NF-κB-dependent signaling in ECs.
(2) (Fig. 2D). Furthermore, TLR3 is well compatible with our observed MyD88 dependency because Alexopoulos et al. (52) initially described TLR3 as MyD88-dependent receptor. We confirm these data by showing that CXCL8/IL-8 induction by the TLR3 agonist poly(I:C) at least partially depends on MyD88. Although we cannot exclude a contribution by TLR3-independent mechanisms, our data demonstrate that TLR3 is involved in mediation of the proinflammatory response induced by the yeast.

In conclusion, our data underscore the importance of primary human ECs as an integrated constituent of the innate immune system in the defense of fungal pathogens and provide novel insights into the molecular mechanisms by which C. albicans elicits exocytic immune responses that under unfavorable conditions can lead to life-threatening sepsis.

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References


