The expression of the β-defensins hBD-2 and hBD-3 is differentially regulated by NF-κB and MAPK/AP-1 pathways in an in vitro model of Candida esophagitis

Nadine Steubesand¹, Karlheinz Kiehne¹, Gabriele Brunke¹, Rene Pahl¹, Karina Reiss³, Karl-Heinz Herzig⁴, Sabine Schubert⁵, Stefan Schreiber¹,², Ulrich R Fölsch¹, Philip Rosenstiel² and Alexander Arlt*¹

Address: ¹Department of Internal Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany, ²Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany, ³Department of Department of Dermatology and Allergology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany, ⁴Institute of Biomedicine, Div of Physiology, and Biocenter of Oulu, University of Oulu, and Department of Internal Medicine, University of Kuopio, Finland and ⁵Institute for Infection Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany

Email: Nadine Steubesand - nadine@steubesand.org; Karlheinz Kiehne - kkiehne@1med.uni-kiel.de; Gabriele Brunke - gbrunke@1med.uni-kiel.de; Rene Pahl - rene.pahl@gmx.de; Karina Reiss - kreiss@dermatology.uni-kiel.de; Karl-Heinz Herzig - Karl-Heinz.Herzig@oulu.fi; Sabine Schubert - schubert@infmed.uni-kiel.de; Stefan Schreiber - s.schreiber@mucosa.de; Ulrich R Fölsch - urfoelsch@1med.uni-kiel.de; Philip Rosenstiel - p.rosenstiel@mucosa.de; Alexander Arlt* - aarlt@1med.uni-kiel.de

* Corresponding author

Abstract

Background: Candida albicans resides on epithelial surfaces as part of the physiological microflora. However, under certain conditions it may cause life-threatening infections like Candida sepsis. Human β-defensins (hBDs) are critical components of host defense at mucosal surfaces and we have recently shown that hBD-2 and hBD-3 are upregulated in Candida esophagitis. We therefore studied the role of Candida signalling pathways in order to understand the mechanisms involved in regulation of hBD-expression by C. albicans. We used the esophageal cell line OE21 and analysed the role of paracrine signals from polymorphonuclear leukocytes (PMN) in an in vitro model of esophageal candidiasis.

Results: Supernatants of C. albicans or indirect coculture with C. albicans induces upregulation of hBD-2 and hBD-3 expression. PMNs strongly amplifies C. albicans-mediated induction of hBDs. By EMSA we demonstrate that C. albicans activates NF-κB and AP-1 in OE21 cells. Inhibition of these pathways revealed that hBD-2 expression is synergistically regulated by both NF-κB and AP-1. In contrast hBD-3 expression is independent of NF-κB and relies solely on an EGFR/MAPK/AP-1-dependent pathway.

Conclusion: Our analysis of signal transduction events demonstrate a functional interaction of epithelial cells with PMNs in response to Candida infection involving divergent signalling events that differentially govern hBD-2 and hBD-3 expression.
**Background**

*Candida albicans* colonize distinct microanatomical regions such as the oro-gastrointestinal tract as a commensal, but also accounts for more than 50% of all fungal systemic infections [1]. *Candida* esophagitis represents a severe threat to an immunocompromised body and, especially in neutropenic patients, often is the first manifestation before *Candida* sepsis develops [2,3]. Host defense mechanisms preventing mucosal (e.g. esophageal) candidiasis are poorly understood, but include both innate and adaptive responses [4,5].

The gastrointestinal epithelial layer represents a barrier that is usually adequate to restrain commensal microbes, but is often insufficient to protect against microbial pathogens. Once this physical barrier is penetrated, recognition of invading microbiota is the first step in the initiation of a fast immune response and involves the activation of pattern recognition receptors by microbial pathogens and their products [6-8]. *C. albicans* have been shown to activate a subset of pattern recognition receptors, the Toll-like receptors (TLRs). This family of transmembrane receptors recognizes a broad variety of signature motifs on microbes and transduces signals leading to the activation of transcription factors [9], production of cytokines and antimicrobial peptides [10,11]. However, there is some controversy and inconclusive data which TLR subtypes are activated by *C. albicans*. Some reports indicate a critical role for TLR2 and TLR4 in activating the host defense response alone or in combination with the ss-glucan receptor Dectin-1, [5,12,13] whereas other studies showed that TLR-1 and TLR-6 are responsible for the recognition of *C. albicans* [14,15].

In addition the downstream effectors in the immune response against *C. albicans* are largely unknown. There is growing evidence that human β-defensins (hBDs) are critical components of both the innate and adaptive immune responses to *Candida* infections with distinct antifungal efficacies and mechanisms for hBD-2 and hBD-3 [16-19]. Expression of hBDs is regulated by a plethora of proinflammatory cytokines like IL-1β, TNF-α and EGF-receptor ligands activating downstream effectors like the transcription factors NF-κB or AP-1 [20-22]. hBDs are secreted by epithelial cells and protect the gastrointestinal mucosa by antimicrobial activity as well as by chemotactic properties recruiting polymorphonuclear leukocytes (PMNs) to the site of infection [21,23]. Transepithelial migration of PMNs is observed during oral *Candida* infection and is believed to play a crucial role in the clearance of infection and in epithelial homeostasis [5,24]. This protective phenotype is associated with production of epithelial proinflammatory cytokines, including IL-8, IL-1β and TNF-α. However, the mechanisms by which PMNs and epithelial cells interact to protect mucosal surfaces from *C. albicans* invasion are mostly unknown.

We have recently shown that hBD-2 and hBD-3 are highly expressed in *Candida* esophagitis and that the α-defensins Human Neutrophil Peptides 1–3 were also upregulated [25] indicating involvement of neutrophils in the immune response to the *C. albicans* infection [26]. This is in line with the observation that IL-8 is strongly expressed in the mucosa of patients suffering by *Candida* infection [25], since this cytokine is involved in recruitment of polymorphonuclear leukocytes (PMNs) to sites of microbial infection [27].

In the present study we explored the mechanisms involved in the induction of hBD expression in an *in vitro* model of *Candida* esophagitis. Using this co-culture model we analyzed the contribution of PMNs in the regulation of epithelial hBD expression. We found that hBD-2 and hBD-3 are strongly upregulated through high concentrations of *C. albicans* alone. If lower concentrations of *C. albicans* were used, only a moderate induction of these hBDs was observed that was strongly enhanced by coculture of the epithelial cell line with PMNs, emphasizing the important role of mesenchymal-epithelial interactions in early host defense against fungal infection. Furthermore we were able to show that the NF-κB and MAPK signalling pathways contribute to the regulation of hBD-2 and that hBD-3 is a downstream target of a distinct EGFR/MAPK/AP-1 pathway in *Candida* esophagitis.

**Results**

**Supernatants of *C. albicans* induce hBD expression in OE21 cells**

To establish an in vitro model for *Candida* esophagitis we first treated several oesophageal cell lines (i.e. OE21, KYSE70, KYSE7150, KYSE7180, KYSE410 and Colo680N) with established inducers of hBD-expression. Only for the OE21 cell line a reliable induction of the mRNA of hBD-2 and hBD-3 could be detected (Figure 1A). We next challenge OE21 cells with supernatants of *C. albicans* cell culture growing under various concentrations of *C. albicans*. Supernatants of *C. albicans* induce upregulation of hBDs in a concentration-dependent manner. For the supernatants of the density of 0.5 × 10³ *C. albicans* cells/ml no significant induction of hBD-2 and hBD-3 was detected (Figure 1B). In contrast the supernatants of 1 × 10⁵ *C. albicans* cells/ml resulted in a significant upregulation of hBD-3 and to a smaller extent of hBD-2 (Figure 1B). hBD1 expression was not altered by one of the inducers or the supernatants (Figure 1).
Supernatants of *C. albicans* induce hBD expression in OE21 cells. OE21 cells were stimulated with A) TNF-α (20 ng/ml), IL-1β (20 ng/ml) or B) supernatants of 0.5 × 10⁴ *C. albicans/ml or 1 × 10⁵ *C. albicans/ml* for 24 h. hBD-1, hBD-2 and hBD-3 gene expression was assessed by real time RT-PCR. Means ± s.d. of three independent experiments performed in triplicate are shown, * indicates p-value <0.05.

**Figure 1**

**Supernatants of *C. albicans* induce hBD expression in OE21 cells.** OE21 cells were stimulated with A) TNF-α (20 ng/ml), IL-1β (20 ng/ml) or B) supernatants of 0.5 × 10⁴ *C. albicans/ml or 1 × 10⁵ *C. albicans/ml* for 24 h. hBD-1, hBD-2 and hBD-3 gene expression was assessed by real time RT-PCR. Means ± s.d. of three independent experiments performed in triplicate are shown, * indicates p-value <0.05.
PMNs significantly enhances hBD-2 and hBD-3 expression during C. albicans infection

To elucidate a potential functional interaction of PMNs and epithelial cells in the immune response to C. albicans infection we established a transwell coculture model. In this two-compartment model we tested the effect of a coculture of OE21 cells, PMNs from healthy donors and C. albicans in different settings: 1) direct contact of OE21 cells with PMNs and 2) direct contact of OE21 cells with C. albicans. The corresponding third component of the system (i.e. C. albicans or PMNs) was cocultured in the transwell system allowing for the interaction of auto- and paracrine factors (Figure 2). Since higher cell numbers than 0.5 × 10⁴ C. albicans cells/ml or 0.5 × 10⁶ PMN/ml repressed growth of OE21 cells in the direct setting (data not shown), all experiments were conducted with these concentrations of C. albicans and/or PMNs. The direct or indirect coculture of OE21 with C. albicans had only small effects on the expression of hBD-2 (Figure 3A) and hBD-3 (Figure 3B) comparable with effects observed with supernatants of the low concentration of C. albicans (Figure 1B). The direct interaction of OE21 cells with PMNs led to an increase of the expression of both hBDs (Figure 3A and Figure 3B). The interaction of OE21 cells with PMNs lead to a significant upregulation of C. albicans induced hBD-2 and hBD-3 expression compared to the induction with C. albicans or PMN alone (Figure 3A and Figure 3B). Substitution of the live C. albicans by supernatants of the density of 0.5 × 10⁴ C. albicans cells/ml also lead to the synergistic effects. To keep the manuscript more concise we only show the data for the live C. albicans.

C. albicans activate NF-κB in OE21 cells

To further analyse the mechanisms involved in the regulation of hBD expression during Candida esophagitis we determined whether the observed induction of hBD-2 and hBD-3 expression by C. albicans was associated with the activation of NF-κB. This transcription factor has been shown to be a central regulator of hBD-2 expression [22,28,29] but there is some controversy if NF-κB is also involved in the regulation of hBD-3 expression [20,30,31]. By EMSA we could show that NF-κB is activated (Figure 4) in association with C. albicans mediated induction of β-defensin expression. In parallel to the observed dependency of defensin expression on the cell number of C. albicans a concentration dependent activation of NF-κB was observed (Figure 4A). The coculture of OE21 cells with PMNs or C. albicans also led to an increased DNA-binding of NF-κB that was further enhanced in the transwell setting (direct PMN and C. albicans indirect; Figure 4B). Supershift assays revealed that the NF-κB complex consisted of the p50 and p65 subunits (Figure 4C).

NF-κB is involved in the induction of hBD-2 but not hBD-3 expression

To evaluate the putative contribution of NF-κB in C. albicans induced hBD expression we transiently transfected cells with siRNA directed against RelA/p65 as the transcriptional active subunit of the p65/p50 NF-κB complex [32]. Transfection of OE21 cells led to a significant reduction of the expression of RelA/p65 (Figure 5A). Furthermore the induction of NF-κB by C. albicans was inhibited by this strategy as shown by EMSA (Figure 5B). This reduction of NF-κB activation strongly reduced the C. albicans mediated induction of hBD-2 expression but had no effect on the hBD-3 expression (Figure 5C). In the coculture settings the reduction of RelA/p65 expression also attenuated hBD-2 induction (data not shown). These results support a critical role of NF-κB in regulating hBD-2 but not hBD-3 expression.

Role of AP-1 and the MAPK Pathway in C. albicans mediated β-defensin induction

Our results indicated a critical role for NF-κB in C. albicans mediated induction of hBD-2 but not for the regulation of hBD-3 expression. Since the hBD-3 promoter contains a functional binding site for the AP-1 transcription factor [33] we conducted EMSA with a probe containing an AP-1 consensus sequence. C. albicans alone (Figure 6A) or in the coculture setting (Figure 6B) activated AP-1 in OE21 cells and supershift experiments identified c-jun as one of the central subunits involved (Figure 6C). Activation of the c-jun/AP-1 complex by the MAPK pathway is well established [34,35] and a recent report indicated a role of this pathway in the response of macrophages to the infection with C. albicans [9]. To analyse the contribution of the MAPK/AP-1 pathway in the induction of β-defensin induction by C. albicans pharmacological inhibitors selective for individual pathway were utilized. As shown in Figure 7A and 7B inhibition of each of the major MAPK pathways resulted in a reduction of hBD-2 and hBD-3 induction by C. albicans.

Activation of the EGFR is an upstream event in regulation of hBD-3 expression

Upstream of the MAPK pathway several growth factor receptors have been shown to be involved in the immune response to microbiota [20,36,37]. To investigate the upstream events leading to the activation of the MAPK pathway in infection with C. albicans we used blocking antibodies against several growth factor receptors. The results revealed a clear involvement of the EGF-receptor in the regulation of hBD-3 expression by C. albicans. Whereas induction of hBD-2 expression was slightly inhibited by a neutralizing antibody targeting EGFR transactivation, a significant reduction of C. albicans-induced hBD-3 expression observed (Figure 8).
PMNs enhances hBD-2 and hBD-3 expression during *C. albicans* infection. OE21 cells were stimulated with $0.5 \times 10^4$ *C. albicans/ml* or $0.5 \times 10^6$ PMNs/ml alone or indicated combinations in a transwell setting for 24 h. Shematic presentation of experimental setting.
PMNs enhances hBD-2 and hBD-3 expression during *C. albicans* infection. OE21 cells were stimulated with 0.5 × 10⁴ *C. albicans/ml or 0.5 × 10⁶ PMNs/ml alone or indicated combinations in a transwell setting for 24 h. A) hBD-2 and B) hBD-3 gene expression was assessed by real time RT-PCR. Means ± s.d. of three independent experiments performed in triplicate are shown, * indicates p-value <0,05, ** indicates synergistic effects with a p-value <0,05.
**Figure 4**

*C. albicans* activate NF-κB in OE21 cells. OE21 cells were stimulated with A) 0.5 × 10⁴ *C. albicans/ml, 1 × 10⁵ *C. albicans/ml, or B) 0.5 × 10⁶ PMNs/ml alone or with direct contact with 0.5 × 10⁶ PMNs/ml and coincubation with 0.5 × 10⁴ *C. albicans/ml in a transwell setting for indicated time periods. Nuclear extracts of these cells were submitted to EMSA using a radiolabelled probe for NF-κB. The arrow indicates the p50/p65 heterodimer. C) Supershift experiments with antibodies directed against the p50 or p65 subunit of NF-κB on nuclear extracts of OE21 cells treated for 1 h with 1 × 10⁵ *C. albicans/ml.
NF-κB is involved in the induction of hBD-2 but not hBD-3 expression. OE21 cells were transfected with a control siRNA or a siRNA directed against RelA/p65. A) Total protein extracts were submitted to Western Blotting for the analysis of RelA/p65 using HSP90 for normalization and B) EMSA on nuclear extracts of siRNA transfected OE21 cells treated with 1 × 10⁵ C. albicans/ml for 1 h. Representative results from three independent experiments are shown. C) SiRNA transfected OE21 cells were incubated with 1 × 10⁵ C. albicans/ml for 24 h and hBD gene expression was assessed by real time RT-PCR. Means ± s.d. of three independent experiments performed in triplicate are shown, * indicates p-value <0.05.
Figure 6
Role of AP-1 and the MAPK Pathway in C. albicans mediated hBD induction. OE21 cells were stimulated with A) $0.5 \times 10^4$ C. albican/ml, $1 \times 10^5$ C. albican/ml, or B) $0.5 \times 10^4$PMNs/ml alone or with direct contact with $0.5 \times 10^4$ PMNs/ml and coincubation with $0.5 \times 10^4$ C. albican/ml in a transwell setting for indicated time periods. Nuclear extracts of these cells were submitted to EMSA using a radiolabelled probe for AP-1. The arrow indicates the AP-1 complex. C) Supershift experiments with an antibody directed against the c-Jun subunit of AP-1 on nuclear extracts of OE21 cells treated for 1 h with $1 \times 10^5$ C. albican/ml.
Figure 7
Role of AP-1 and the MAPK Pathway in C. albicans mediated hBD induction. OE21 cells were preincubated with inhibitors for MEK (PD98059, 10 μM), p38 (SB203580 10 μM) and JNK (SP600125 20 μM) kinase pathways for 1 h and then incubated with direct contact with 0.5 × 10⁶ PMNs/ml and coincubation with 0.5 × 10⁴ C. albicans/ml in a transwell setting for additional 24 h. A) hBD-2 and B) hBD-3 gene expression was assessed by real time RT-PCR. Means ± s.d. of three independent experiments performed in triplicate are shown, * indicates p-value <0.05.
Discussion

Candida esophagitis represents a severe threat to an immunocompromised body and the course of the infection is determined by both pathogen- and host-dependent factors [15,24,38,39]. It is well established that epithelial cells of the esophagus are the central target of an oro-esophageal invasive Candida infection but there are only very limited data on the host response preventing a Candida esophagitis. In the present study we identified the NF-κB and MAPK/AP-1 pathways as central regulators of epithelial hBD-2 and hBD-3 expression during C. albicans infection. Furthermore we were able to show a crucial role of the interaction of PMNs with the epithelial cell compartment for the induction of hBD-2 and hBD-3 expression during Candida infection of esophageal epithelial cells. Finally hBD-3 expression is dependent on transactivation of EGFR by TGF-α. This is the first report delineating molecular mechanisms leading to upregulation of hBD-2 and hBD-3 in esophageal Candida infection suggesting differential regulation of these two central epithelial antibiotic peptides in response to a C. albicans infection. We were able to show a concentration dependent upregulation of hBD-3 expression (Figure 9).

TGF-α is involved in EGFR mediated hBD-3 induction

Finally we searched for the ligand activating the EGFR. By using blocking antibodies for the established EGFR-ligands EGF, HB-EGF, amphiregulin and TGF-α we were able to show that TGF-α is involved in the EGFR mediated upregulation of hBD-3 expression (Figure 9).

Figure 8

Activation of the EGFR is an upstream event in regulation of hBD-3 expression. OE21 cells were preincubated with a blocking EGFR antibody (10 μg/ml) for 1 h and then incubated with direct contact with 0.5 × 10⁶ PMNs/ml and coinoculation with 0.5 × 10⁶ C. albicans/ml in a transwell setting for additional 24 h. hBD gene expression was assessed by real time RT-PCR. Means ± s.d. of three independent experiments performed in triplicate are shown. * indicates p-value <0.05.

There is some controversy about the relevance of the NF-κB and AP-1 binding sites in the promotors of hBD-2 for full induction of hBD-2 expression after treatment with IL-1β or infection with microbiota. Wehkamp and colleagues showed that the parallel activation of NF-κB and AP-1 is needed for full transcriptional activation of the hBD-2-promotor after IL-1β stimulation, treatment of keratinocytes with supernatants of Pseudomonas aeruginosa [22], or E. coli mediated hBD-2 induction [41]. On the other hand another study demonstrated induction of hBD-2 expression through Fusobacterium nucleatum in human gingival epithelial cells independent of NF-κB activation [42] and there is also evidence for induction of hBD-2 through Salmonella enteritidis [43] or H. pylori [44] in the absence of functional AP-1. In the present work we were able to show that both NF-κB and AP-1 activation are required for full upregulation of the hBD-2 mRNA after treatment with either supernatants of C. albicans or the coculture of C. albicans with PMNs. Inhibition of the NF-κB or of the MAPK/AP-1 pathway significantly reduced the induction of hBD-2 expression confirming the central role of both transcription factors. These observations are in line with recent results for H. pylori infection [20] and the effects of lactobacilli and the VSL#3 bacterial mixture on enterocytes [45]. The role of NF-κB is less clear in the regulation of hBD-3 expression. In contrast to a recently reported role of NF-κB in the regulation of the hBD-3 gene in keratinocytes [31] the majority of data failed to show a functional relevance for NF-κB in controlling hBD-3 expression [33,46]. By using siRNA targeting the RelA/p65 subunit of NF-κB we could demonstrate that C. albicans mediates hBD-3 upregulation through a MAPK/AP-1 pathway independently of the observed NF-κB activation. To investigate the proposed role of PMNs in the immune response the C. albicans infection [25,26] we established an in vitro model (Figure 2). PMNs alone induced NF-κB and AP-1 leading to hBD-2 and hBD-3 expression. Coincubation of PMNs with C. albicans lead to a significant upregulation of hBD-2 and hBD-3 expression compared to effects of PMNs or C. albicans alone. Inhibition of the MAPK/AP-1 pathway reduced the expression of both hBDS under this condition. The fact that PMNs alone induced hBD expression indicated that PMNs could contribute to the induction of antimicrobial peptides in epithelia during inflammation.
TGF-α is involved in EGFR mediated hBD-3 induction. OE21 cells were preincubated with the blocking EGFR antibody (10 μg/ml) or neutralizing antibody against EGF (0.5 μg/ml), HB-EGF (10 μg/ml), amphiregulin (10 μg/ml) or TGF-α (1 μg/ml) for 1 h and then incubated with direct contact with 0.5 × 10⁶ PMNs/ml and coincubation with 0.5 × 10⁴ C. albican/ml in a transwell setting for additional 24 h. hBD gene expression was assessed by real time RT-PCR. Means ± s.d. of three independent experiments performed in triplicate are shown, * indicates p-value <0.05.
There is some evidence that a TLR-4 mediated interplay between PMNs and epithelial cells is important for the protective response against Candida infections [5] but the exact mechanisms remained elusive. One the one hand PMNs induced expression and release of IL-6 and IL-8 in O21 cells (Additional file 1) which might in turn activate NF-κB and AP-1 leading to hBD-2 and hBD-3 expression. On the other hand the induction of a TH1 response, i.e. TNF-α and interferon-γ might directly induce or enhance the expression of hBDs as reported recently [47]. On the other hand PMNs are able to establish an anti-fungal response by upregulate the expression of TLR4 in epithelial cells [5]. The observed more pronounced effect on hBD-3 expression are in line with the theory that hBD-3 might be clinically more relevant than hBD-2 since hBD-2 and hBD-3 have potent fungicidal activity against C. albicans at micromolar concentrations, with hBD-3 being about 10 times more fungicidal than hBD-2 [16,17,19]. Finally we demonstrated that hBD-3 expression was induced by transactivation of the EGFR independent of EGF (Figure 8 and 9). Many signals besides EGF converge to inhibit the induction of hBD-3 expression (Figure 9). Since we are also able to show that most likely TGF-α is involved in the EGFR mediated upregulation of hBD-3 expression (Figure 9). Since we are also able to inhibit the induction of hBD3 by the supernatants of 1 × 10^5 C. albicans cells/ml by EGFR and/or TGF-α blocking antibodies we speculate that the EGFR-ligand is secreted by OE21 cells. Ligands of the EGFR are expressed as transmembrane precursors. These are released from the cell surface following shedding of the extracellular domain by a family of metalloproteinases (a disintegrin and metalloprotease (ADAM)). ADAM10, -12 and -17 are the sheddases of the EGFR ligands in response to various stimuli. Since human cathelicidin cationic antimicrobial protein (hCAP)-18 and its active peptide LL-37 have been shown to be involved in the transactivation of the EGFR at the airway epithelial surfaces [50,51] it is tempting to speculate that the activation of PMNs by C. albicans leads to secretion of proinflammatory mediators including leukotriene B4 (LTB4) and LL-37 [52,53] which in turn amplifies the inflammatory response [54,55] and leads to the shedding of EGFR-ligands.

In conclusion we were able to establish a complex in vitro model for Candida infection of esophageal cells to investigate the signalling events leading to upregulation of hBD-2 and hBD-3 expression.

**Conclusion**

This analysis of signal transduction events in esophageal Candida infection demonstrated a potential functional interaction of epithelial cells with PMNs and that EGFR, NF-κB and MAPK/AP-1 are involved in divergent signalling events governing hBD-2 and hBD-3 expression. Especially the observed effect of PMNs on hBD expression might explain the high incidence of Candida esophagitis and Candida related deaths in neutropenic patients.

**Methods**

**Materials**

Cell culture medium was purchased from PAA-Labs (Linz, Austria), fetal bovine serum from Seromed (Berlin, Germany), recombinant TNF-α from Sigma. Supershift and Westernblot p65, p50 and c-Jun antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Organism and Cell culture**

OE21 cells, a moderately differentiated oesophageal squamous carcinoma cell line were cultured in RPMI 1670 supplemented with 10% fetal calf serum and divided every 3 days. Cells for experimental purposes were cultured in 6 well cell culture plates with the same medium and used at subconfluence. C. albicans (a clinical isolate from oesophagitis [25]) was taken from a frozen strain suspension (BHI, glycerine). 10^6 cells was transferred into 10 ml TSB-Bouillon and incubated overnight at 37°C. The cells were then separated from the medium by centrifugation at 2800 × g for 5 min, washed two times with PBS, and finally resuspended in RPMI for each experiment. The final concentration of C. albicans was 1 × 10^4/ml.

**Inhibitor studies**

OE21 cells were pre-treated with specific inhibitors or neutralizing antibodies for 30–60 min prior to and during Candida or cytokine stimulation. The neutralizing antibodies against the EGFR (Upstate, USA), TGF-α (R&D Systems), HB-EGF (R&D Systems), amphiregulin (R&D Systems), and EGF (Pepro Tech, Germany) were used at the indicated concentrations. Inhibitors utilized were: PD98059 (Calbiochem) at 10 μM, SB203580 (Tocris, Ellusville, USA) at 10 μM, SP600125 (Tocris, Ellusville, USA) at 20 μM. Inhibitor were dissolved in DMSO and DMSO was used as a vehicel control.

**Generation of Supernatants from C. albicans**

To analyze the effects of secreted factors from C. albicans on OE21 cells, C. albicans were cultured with respective medium for 48 h in the indicated density (0.5 × 10^4 to 1 × 10^5 cells/ml). Before use, these conditioned media were precleared by centrifugation (10,000 rpm for 10 min) and used for additional experiments.
Isolation of polymorphonuclear leukocytes (PMN)

PMNs were purified using LeucoSep tubes according to the instructions of the manufacturer (Greiner Bio-One). In brief, 3 ml of Ficoll-Paque was preloaded in a 14 ml LeucoSep tube by centrifugation for 30 s at 1,000 g. The heparinized whole-blood samples of healthy volunteers were diluted with equal volumes of PBS, and 6 ml of the diluted blood was added to a LeucoSep tube. The cell separation tubes were centrifuged for 15 min at 800 g without braking at room temperature. The cell suspension was collected, and the cells were washed twice in PBS (for 10 min at 640 and 470 g, respectively, for the two successive wash steps) and resuspended in complete RPMI medium before counting.

Stimulation with Supernatants, direct cell contact and transwell coculture model

2 × 10⁵ OE21 cells were seeded into a six-well culture plate. For stimulation experiments medium was replaced after 24 h with the C. albicans supernatants and cultured for indicated times. For direct cell contact suspension cultures of C. albicans (final concentration 0.5 × 10⁴ cells/ml) or PMNs (final concentration 0.5 × 10⁶ cells/ml) were added to the OE21 cells after initial 24 h culture and incubated for the indicated times. In the transwell setting (for a schematic representation please refer to Figure 2) 2 × 10⁵ OE21 cells were cultured into the bottom compartment of a six-well culture plate. After 24 h PMNs or C. albicans were added to the bottom compartment and the corresponding cell type was seeded into the top transwell compartment (Costar GmbH, Bodenheim, Germany) and cultured for additional 24 h.

RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy Kit from Qiagen (Hilden, Germany) and reverse-transcribed into single-stranded cDNA, as described previously [56].

Primer

The oligonucleotide sequence and product size for each primer pair used were as described previously [25].

Quantification of gene expression by real time PCR

Real-time PCR analyses were performed as previously described [56]. Standard curves for each mRNA were constructed by cloning the purified PCR-products containing the target sequence into pCR-Blunt II-TOPO vector (Invitrogen). Concentration of the reference plasmid was measured spectrophotometrically and transformed into number of copies/μl by calculation. The absolute mRNA transcript number in each sample was calculated by use of calibration curves. Identical results were obtained in control experiments.

Cytokine profiling

Culture supernatants were cleared by centrifugation (6,000 rpm, 5 min, 4°C) and protein concentration was determined by the BioRad assays. For a qualitative screening for the cytokine content, samples were adjusted to equal protein concentration and submitted to cytokine antibody arrays (Cytokine Profiler kit, R&D systems) following the manufacturer’s instructions.

siRNA transfection

For knock down of RelA/p65, cells were seeded into 6 well plates (2 × 10⁵ cells/well) and grown overnight, then transfection with 12 μl/well RNAiFect reagent (Invitrogen) and 2 μg/well of either Stealth negative control siRNA (Invitrogen) or Stealth RelA/p65 siRNA (Invitrogen) was performed for 48 h.

Western Blotting

Cellular lysates were prepared and western blotting was performed as described previously [57,58].

EMSA

Nuclear extracts were prepared as described previously [58] and incubated with a γ32P-labelled oligonucleotide containing a consensus NF-κB-binding or consensus AP-1 site (Promega; Mannheim, FRG). After 30 min incubation at room temperature, samples were separated by gel electrophoresis at 100 V and 4°C. Gels were dried and exposed to X-ray Hyperfilm (Amersham; Freiburg, FRG).

Statistics

Data are presented as mean ± SD and analyzed by Student’s t-test. A p-value < 0.05 (indicated as * in the figures) was considered as statistically significant.

Authors’ contributions

NS, GB, RP and KK carried out the experimental studies and drafted the manuscript. KR, RP, KHH, SS, URF and PR participated in the design of the study and performed the statistical analysis. AA conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

PMNs induce IL-6 and IL-8 in OE21 cells. Supernatants of OE21 cells kept in culture alone (upper panel) or with with direct contact with 0.5 × 10⁶ PMNs/ml (lower panel) for 24 h were submitted to a peptide arrays detecting a broad panel of chemokines and cytokines.
Acknowledgements
The authors would like to thank Silvia Voss, Christiane Sandberg, Cornelia Wilgus and Frauke Grohmann for excellent technical support. This work is part of a PhD thesis (N.S.) and a MD thesis (R.P.). This study was supported by grants of the German Research Society (DFG/SFB617) to K.K., P.R. and A.A.

References
1. Tortorano AM, Kibbler C, Peman J, Bernhardt H, Klingspor L, Grillot R: Candidaemia in Europe: epidemiology and resistance. Int J Antimicrob Agents 2006, 27:359-366.
2. Jensen T, Obel N: Preserved neutrophil response to Candida albicans stimulation in AIDS patients with candida esophagitis. Apmis 1996, 104:591-597.
3. Gornig M, Emmert-Buck M, Walsh TJ: [Patterns of Candida esophagitis in cancer and AIDS patients: histopathological study of 23 patients]. Mycoses 1997, 40(Supp1):81-85.
4. Vilanova M, Correia A: Host defense mechanisms in invasive candidiasis originating in the GI tract. Expert Rev Anti Infect Ther 2008, 6:441-445.
5. Weindl G, Naglik JR, Kaesler S, Biedermann T, Hube B, Korting HC, Schaller M: Human epithelial cells establish direct antifungal defense through TLR4-mediated signaling. J Clin Invest 2007, 117:3664-3672.
6. Trinchieri G, Sher A: Cooperation of Toll-like receptor signals in innate immune defense. Nat Rev Immunol 2007, 7:179-190.
7. Rosenstiel P, Till A, Schreiber S: NOD-like receptors and human diseases. Microbes Infect 2007, 9:648-657.
8. West TE, Ernst RK, Janssen-Hutson MJ, Skerritt SJ: Activation of Toll-like receptors by Burkholderia pseudomallei. BMC Immunol 2008, 9:24.
9. Roeder A, Kirschning CJ, Schaller M, Weinid G, Wagner H, Korting HC, Rupec RA: Induction of nuclear factor-kappa B and c-Jun/activator protein-1 via toll-like receptor 2 in macrophages by antymycotic-treated Candida albicans. J Infect Dis 2004, 190:1118-1126.
10. Froy O: Regulation of mammalian defensin expression by Toll-like receptor-dependent and independent signalling pathways. Cell Microbiol 2005, 7:1387-1397.
11. Karlsson J, Putsep K, Chu H, Kays RJ, Bevins CL, Andersson M: Regional variations in Paneth cell antimicrobial peptide expression along the mouse intestinal tract. BMC Immunol 2008, 9:37.
12. Netea MG, Graaf CA Van Der, Vonk AG, Verschueren I, Meer JW Van Der, Kullberg BJ: The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. J Infect Dis 2002, 185:1483-1489.
13. Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, Adema GJ: Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cell Immunol 2007, 258:2058-2066.
14. Saegusa S, Totsuka M, Kamingawa S, Hosoi T: Candida albicans and Saccharomyces cerevisiae induce interleukin-8 production from intestinal epithelial-like Caco-2 cells in the presence of butyric acid. FEMS Immunol Med Microbiol 2004, 41:227-234.
15. Netea MG, Veerdonk F van de, Verschueren I, Meer JW van der, Kullberg BJ: Role of TLR1 and TLR6 in the host defense against disseminated candidiasis. FEMS Immunol Med Microbiol 2008, 52:118-123.
16. Vylkova S, Nayyar N, Li W, Edgerton M: Human beta-defensins kill Candida albicans in an energy-dependent and salt-sensitive manner without causing membrane disruption. Antimicrob Agents Chemother 2007, 51:154-161.
17. Vylkova S, Li XS, Berner JC, Edgerton M: Distinct antifungal mechanisms: beta-defensins require Candida albicans Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. Antimicrob Agents Chemother 2006, 50:324-331.
18. Feng Z, Jiang B, Chandra J, Ghannoum M, Nelson S, Weinberg A: Human beta-defensins: differential activity against candidal species and regulation by Candida albicans. J Dent Res 2005, 84:445-450.
19. Joly S, Maze C, McCray PB Jr, Guthmiller JM: Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. J Clin Microbiol 2005, 43:1021-1029.
20. Bouguen PK, Argent RE, Body-Malapel M, Park JH, Ewings KE, Bowie AG, Ong Sj, Cook Sj, Sorense ON, Manzo BA, et al.: Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during Helicobacter pylori infection. J Biol Chem 2006, 281:11637-11648.
21. Wehkamp J, Schaufer J, Stange EF: Defensins and cathelicidins in gastrointestinal infections. Curr Opin Gastroenterol 2007, 23:32-38.
22. Wehkamp K, Schwichtenberg L, Schroder JM, Harder J: Pseudomonas aeruginosa- and IL-1beta-mediated induction of human beta-defensin-2 in keratinocytes is controlled by NF-kappaB and AP-1. J Invest Dermatol 2006, 126:121-127.
23. Bowdish DM, Davidson DJ, Hancock RE: Immunomodulatory properties of defensins and cathelicidins. Curr Top Microbiol Immunol 2006, 306:27-66.
24. Schaller M, Zaklikhany N, Naglik JR, Weinid G, Hube B: Models of oral and vaginal candidiasis based on in vitro reconstituted human epithelia. Nat Protoc 2006, 1:2767-2773.
25. Kiesehe K, Brunke G, Nave T, Pfeffer E, Hintzen KH: Oesophageal defensin expression during Candida infection and reflux disease. Scand J Gastroenterol 2005, 40:501-507.
26. Cunliffe RN: Alpha-defensins in the gastrointestinal tract. Mol Immunol 2003, 40:463-467.
27. Godaly G, Bergsten G, Hang L, Fischer H, Frendeus B, Lundstedt AC, Samuelsson M, Samuelsson P, Svannborg C: Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection. J Leukoc Biol 2001, 69:899-906.
28. Moon SK, Lee HY, Pan H, Takeshita T, Park R, Cha K, Andalibi A, Lim DJ: Synergetic effect of interleukin 1 on nontypeable Haemophilus influenzae-induced up-regulation of human beta-defensin 2 in middle ear epithelial cells. BMC Infect Dis 2006, 6:12.
29. Wang X, Zhang Z, Louboutin JP, Moser C, Weiner DJ, Wilson JM: Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. Faseb J 2003, 17:1772-1779.
30. Sayama K, Komatsuzawa H, Yamasaki K, Shirakata Y, Hangakaya Y, Obara K, Tokumura S, Dai X, Tohyama M, Ten Dijke P, et al.: New mechanisms of skin innate immunity: ASK1-mediated keratinocyte differentiation regulates the expression of beta-defensins, LL37, and TLR2. Eur J Immunol 2005, 35:1886-1895.
31. Albanesi C, Fairchild HR, Madonna S, Scarpioni C, De Pita O, Leung DT, Howell MD: IL-4 and IL-13 negatively regulate TNF-alpha and IFN-gamma-induced beta-defensin expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3. J Immunol 2007, 179:984-992.
32. Perkins ND: Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene 2006, 25:6717-6730.
33. Menzies BE, Kenoyer A: Signal transduction and nuclear responses in Staphylococcus aureus-induced expression of human beta-defensin 3 in skin keratinocytes. Infect Immun 2006, 74:6847-6854.
34. Whitmarsh AJ: Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. Biochim Biophys Acta 2007, 1773:1285-1298.
35. Turjanski AG, Vaqe JP, Gudtik J: MAP kinases and the control of nuclear events. Oncogene 2007, 26:3420-3435.
36. Miller LS, Sorensen OE, Liu PT, Julian HR, Eshaghpour D, Behmanesh BE, Chung W, Starner TD, Kim J, Sieling PA, et al.: TGF-alpha regulates TLR expression and function on epidermal keratinocytes. J Immunol 2005, 174:6137-6143.
37. Sorensen OE, Thiapa DR, Rosenthal A, Liu L, Roberts AA, Ganz T: Differential regulation of beta-defensin expression in human skin by microbial stimuli. J Immunol 2005, 174:4870-4879.
38. Barker KS, Park H, Phan QT, Xu L, Homayouni R, Rogers PD, Filler SG: Transcripome profile of the vascular endothelial cell response to Candida albicans. J Infect Dis 2008, 198:193-202.
39. Muller V, Viemann D, Schmidt M, Enares N, Ludwig S, Leverkus M, Roth J, Goebeler M: Candida albicans triggers activation of distinct signaling pathways to establish a proinflammatory gene expression program in primary human endothelial cells. J Immunol 2007, 179:8435-8445.
40. Inoue Y, Matsuwaki Y, Shin SH, Ponikau JU, Kita H: Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. J Immunol 2003, 170:5439-5447.

41. Wahlkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schleee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, et al.: NF-kappa B- and AP-1-mediated induction of human beta-defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. Infect Immun 2004, 72:5750-5758.

42. Krisanaprakornkit S, Kimball JR, Dale BA: Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappa B transcription factor family. J Immunol 2002, 168:316-324.

43. Ogushi K, Wada A, Niidome T, Mori N, Oishi K, Nagatake T, Takahashi A, Assakura H, Makino S, Hojo H, et al.: Salmonella enteritidis FlIC (flagella filament protein) induces human beta-defensin-2 mRNA production by Caco-2 cells. J Biol Chem 2001, 276:30521-30526.

44. Wada A, Ogushi K, Kimura T, Hojo H, Mori N, Suzuki S, Kumatori A, Se M, Nakahara Y, Nakamura M, et al.: Helicobacter pylori-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappa B. Cell Microbiol 2001, 3:115-123.

45. Schlee M, Harder J, Koter B, Stange EF, Wehkamp J, Fellermann K: Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clin Exp Immunol 2008, 151:528-535.

46. Chung WO, Dale BA: Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species. Infect Immun 2004, 72:352-358.

47. Joly S, Organ CC, Johnson GK, McCray PB Jr, Guthmiller JM: Correlation between beta-defensin expression and induction profiles in gingival keratinocytes. Mol Immunol 2005, 42:1073-1084.

48. Koff K, Shao MX, Ueki IF, Nadel JA: Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. Am J Physiol Lung Cell Mol Physiol 2008, 294:L1068-1075.

49. Pastore S, Mascia F, Mariani V, Girolomoni G: The epidermal growth factor receptor system in skin repair and inflammation. J Invest Dermatol 2008, 128:1365-1374.

50. Zuyderduyn S, Ninaber DK, Hiemstra PS, Rabe KF: The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells. J Allergy Clin Immunol 2006, 117:1328-1335.

51. Tjibbringa GS, Aarbouw J, Ninaber DK, Drijfhuizen JW, Sorensen OE, Borregaard N, Rabe KF, Hiemstra PS: The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. J Immunol 2003, 171:6690-6696.

52. Flamand L, Tremblay MJ, Borgeat P: Leukotriene B4 triggers the in vitro and in vivo release of potent antimicrobial agents. J Immunol 2007, 178:8036-8045.

53. Wan M, Sabirah A, Wetterholm A, Agerberth B, Haggestrom JZ: Leukotriene B4 triggers release of the cathelicidin LL-37 from human neutrophils: novel lipid-peptide interactions in innate immune responses. Faseb J 2007, 21:2897-2905.

54. Soehnlein O, Zernecke A, Eriksson EE, Rothfuchs AG, Pham CT, Heward H, Bidzhekov K, Rottenberg ME, Weber C, Lindbom L: Neutrophil secretion products pave the way for inflammatory monocytes. Blood 2008, 112:1461-1471.

55. de Haar SF, Hiemstra PS, van Steenbergen MT, Everts V, Beertsen W: Role of polymorphonuclear leukocyte-derived serine proteases in defense against Actinobacillus actinomycetemcomitans. Infect Immun 2006, 74:5284-5291.

56. Kiehn K, Fincke A, Brunk E, Lange T, Folsch UR, Herzog KH: Anti-microbial peptides in chronic anal fistula epithelium. Scand J Gastroenterol 2007, 42:1063-1069.

57. Arlt A, Rosenstiel P, Krut A, Mohn M, Grohmann F, Minkenberg J, Perkins ND, Folsch UR, Schreiber S, Schafer H: IEX-1 directly interferes with RelA/p65 dependent transactivation and regulation of apoptosis. Biochim Biophys Acta 2008, 1783:941-952.

58. Sebens Muirkoster S, Rausch AV, Ibsener A, Minkenberg J, Blaszczyk E, Witt M, Folsch UR, Schmitz F, Schafer H, Arlt A: The apoptosis-induced effect of gastrin on colorectal cancer cells relates to an increased IEX-1 expression mediating NF-kappa B inhibition. Oncogene 2008, 27:1122-1134.