International Journal of Medical Microbiology 301 (2011) 384–389

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.de/ijmm

Mini Review

Adaptation, adhesion and invasion during interaction of *Candida albicans* with the host – Focus on the function of cell wall proteins

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ARTICLE INFO

Keywords:
Candida albicans
Host–pattern interaction
Transcriptional profiling
Cell wall
Pathogen recognition receptors

ABSTRACT

Infectious diseases have long been regarded as losing their threat to mankind. However, in the recent decades infectious diseases have been regaining grounds and are back in the focus of research. This is also due to the fact that medical progress has enabled us to treat and cure a much higher fraction of severe diseases or trauma, resulting in a significant proportion of temporarily or constantly immune-suppressed patients. Infectious diseases result from the interplay between pathogenic microorganisms and the hosts they infect, especially their defense systems. Consequently, immune-suppressed patients are at high risk to succumb from opportunistic infections, like *Candida* infections. To study the balance between host and *C. albicans* with regard to the establishment of disease or asymptomatic, commensal colonisation, we developed host–pathogen interaction systems to study both the adaptation of *C. albicans* to different epithelia as well as to investigate the sensors of the innate immune system, the pattern recognition receptors. These host–pathogen interaction systems, as well as some of the results gained are described in this review.

Introduction

Candida albicans is the most frequent causative agent of severe systemic fungal infections of humans. As a commensal organism, present in the gastrointestinal or urogenital tract of 30–60% of the population, this opportunistic pathogen is widely spread and a constant risk for immuno-compromised patients especially in intensive care units. *C. albicans* occurs in a variety of warm blooded animals, however, unlike *Aspergillus* spp. it is in general not found in the environment. This indicates that this organism must have adapted very well to its hosts in order to persist and proliferate. Indeed, *C. albicans* is able to adhere to a variety of different tissues within the human body thus facilitating the occupation of many host niches. Remarkably, such host niches provide very different environments for growth (pH, O_2 levels, temperature, nutrient availability). This suggests that *C. albicans* must have developed a large array of signaling and adaptation mechanisms in order to persist and proliferate in these environments, rendering *C. albicans* such an effective colonizer of the human body eventually causing disease (Calderone, 2002). To identify key mechanisms of *C. albicans* critical for colonization and infection of the host, the development of host–pathogen models is crucial (Casadevall and Pirofski, 2001). Therefore, infection models being most similar to the host and simple to handle as well as the technologies to analyze the molecular mechanisms of host–pathogen interaction are crucial for understanding pathogenesis. We developed a set of *in vitro* tissue models and genome-wide technologies to analyze host–pathogen interaction. This review will give a short overview of the approaches and results achieved employing these tissue models and their molecular analytics in our lab.

Host–pathogen interaction models

To understand how *C. albicans* is able to persist at and infect virtually any body site, several different models for host–pathogen interaction have been developed and are currently used in a wide variety of interaction studies. This includes *in vivo* models like mice, *Caenorhabditis elegans* (Mylonakis et al., 2003), *Drosophila melanogaster* (Ferrandon et al., 2004; Mylonakis and Aballay, 2005) and *Galleria mellonella* (Cotter et al., 2000) as well as cell lines derived from different body sites or immune cells like macrophages, dendritic cells and neutrophils (both primary cells and cell lines). Recently, some of these models have been reviewed and a collection of protocols for their application was summarized (Rupp and Sohn, 2009). *In vitro* or *ex vivo* models have the advantage to enable thorough molecular analyses at any time point of

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doi:10.1016/j.ijmm.2011.04.004

infection. However, they are in general artificial systems lacking major components of the host like an immune system or, if isolated immune cells are used, lacking their physiological environment. Nevertheless these models have been tremendously useful in delineating mechanisms of host–pathogen interaction. The enterocytic Caco-2 (colon adenocarcinoma derived cell line), A-431 (derived form vulvo-vaginal carcinoma), FaDu (oral epithelial cell line from a pharyngeal carcinoma) and the TR-146 cell line derived from squamous carcinoma of buccal mucosa are among the most frequently used cell lines for setting up simple infection models, representing the respective body site (Dalle et al., 2010; Dieterich et al., 2002; Hernandez and Rupp, 2009; Park et al., 2009; Sohn and Rupp, 2009; Sohn et al., 2006b; Spiering et al., 2010; Zhu and Filler, 2010). Some of the cell line-based models are commercially available and have been termed reconstituted human epithelia (RHE), by the manufacturer (e.g. SkinEthic Laboratories, France). More complex models consisting of more than one human cell line or primary cells have also been established as infection models. These include for example skin models consisting of primary keratinocytes growing on a collagen matrix containing dermal fibroblasts (Dieterich et al., 2002) as well as RHE to which neutrophils (PMNs) have been added (Schaller et al., 2004). Furthermore, explanted endothelial cells as well as endothelial cell lines and *ex vivo* tissues of different origin up to explanted organs like swine liver or intestines as well as adult mouse colon have been developed as infection models (Bareiss et al., 2008; Dixon, 1987; Filler et al., 1995; Mayer et al., 1992; Thewes et al., 2007; Wendland et al., 2006).

Identification of cell surface genes relevant for host–pathogen interaction

The cell wall is the prime interaction site between fungal pathogens and the host. It contains a multitude of proteins required for adhesion to the host, immune evasion and structural integrity. It is also an essential structure that is characteristic for all fungi, not present in mammals, thus it is a prime target for antimycotics (Sohn et al., 2006a). In fact, one class of antimycotics, the echinocandins, directly target the cell wall (by inhibiting beta-(1,3)-D-glucan synthesis) acting fungicidal against most *Candida* spp. and fungistatic against *Aspergillus* spp. (Denning, 2003). Therefore, the cell wall structure and its protein constituents are highly relevant for elucidating host–pathogen interaction mechanisms and the identification of drug targets; and are in the focus of intensive studies (for review see Chaffin, 2008; Klis et al., 2009). The cell wall hosts a variety of different proteins, both covalently attached for example via GPI-anchors (De Groot et al., 2003), but also non-covalently linked cell surface proteins which may eventually be secreted (e.g. Pra1p, Sun41p as described below) or have been shown to be involved in cell wall biogenesis, like Tsa1p (Urban et al., 2005). An array of distinct cell wall proteins required for adhesion to inert or biological surfaces including biofilm formation has been described. This includes, among others adhesins, a set of at least eight ALS proteins (Hoyer et al., 2008) which act as agglutinins under different environmental conditions. For another protein, Hwp1p, it was shown that it serves as a transglutaminase substrate resulting in a covalent hook up of *C. albicans* to epithelia (Staab et al., 1999). This diversity of proteins and the sophisticated mechanisms to attach to surfaces give an idea about the efficiency of *C. albicans* in colonizing host surfaces. The expression and localisation of these proteins are governed by a multitude of signaling pathways and depend on an intact cell wall structure. Since cell surface proteins are inherently difficult to characterize on a protein level, due to their covalent linkage to the cell surface and their often high post-translational modifications, transcriptional profiling is another option to both look at the transcriptional changes of cell surface proteins on a genome wide level as well as changes in their regulatory mechanisms.

Transcriptional profiling is one of the most common methods to generate a holistic view of gene expression levels at a defined condition. By comparing the expression profiles between two or several conditions, the alterations in transcription required by the organism to adapt to the new condition(s) can be delineated. If a defined reference condition is used, this reference condition will have a significant impact on the results. For example if the medium needs to be changed between reference and experiment, all genes required to adapt to the new medium condition will be modulated. The differences in setting these parameters certainly contribute to the large heterogeneity observed throughout the current data sets available in the literature. Also, distinct methods for data analysis may result in variations (Hauser et al., 2009). Different array formats may additionally contribute to differences in the results obtained (for review see Rupp, 2008). We have developed partial and genome-wide DNA-microarrays based on assembly 6 of the *Candida* genome (Hauser et al., 2002; Sohn et al., 2003) (http://www-sequence.stanford.edu/group/candida), which were the basis for our analysis of the *Candida*-epithelia interaction (Sohn et al., 2006b). This array was recently completely revised and updated to assembly 21 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nfyhjmusyqsgqzo&acc=GPL10374).

Transcriptional response of *C. albicans* during adherence to different epithelia

In order to address the question if *C. albicans* is able to adapt specifically to or is affected differentially by distinct epithelia we analyzed the transcriptional profiles of *C. albicans* in suspension culture, on Caco-2 and A-431 cells and compared them to the profiles of *C. albicans* grown on abiotic surfaces, e.g. polystyrene (Sohn et al., 2006b). By using exactly identical conditions except for the surface available for growth, we expected to eliminate most media-derived effects like induction of hyphal genes due to transition from rich, or very poor media to cell culture media. The primary focus of these experiments was to look at the initial phase of adhesion up to the beginning of invasion. These phases of colonisation and infection may be reflected in these simple cell line-derived infection models in a way similar to an *in vivo* situation whereas later time points, including strongly invasive stages could deviate significantly, due to the lack of an immune system in these simplified models. To determine the optimal time points for sample preparation we determined the time required for adhesion of *C. albicans* to the different surfaces and visualized the initial attachment and beginning penetration into the tissue using scanning electron microscopy (SEM) and histology (Fig. 1). Based on these analyses we chose four time points from 30 min (~50% adhesion) up to 240 min showing beginning invasion. The overall results confirmed that *C. albicans* indeed reacts to different surfaces by distinct gene expression profiles. The general changes in transcript levels were moderate, not exceeding 4-fold. Another general trend was the reduction of transcript levels for ribosomal proteins, indicating a stress response if compared to the bare polystyrol surface, as well as the induction of gluconeogenetic and nitrogen limitation response genes. This includes key metabolic functionalities like *ICL1, FBP1* and *GCN4*, but also genes involved in pathogenesis like *ALS1* or some of the SAP genes and genes related to oxidative stress response like *TSA1, GRP2* and *GPX2* (these data are available on www.domchips.org). Some of the genes encoding cell wall proteins identified in this study are described in detail below. Zakikhany et al. (2007), using oral epithelia as infection models as well as samples from patients with pseudomembranous oropharyngeal candidiasis, identified both an overlapping as well as a distinct set

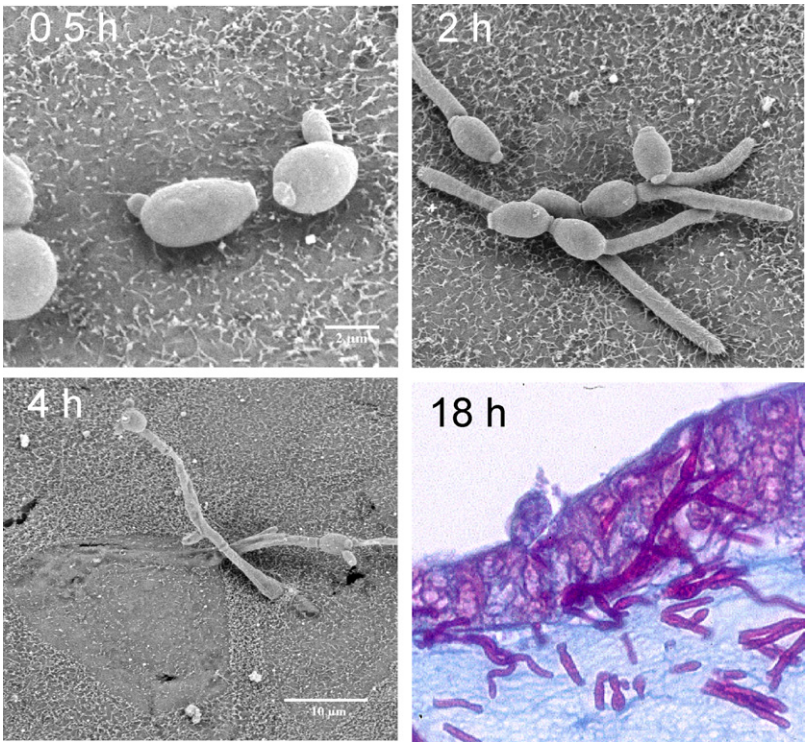

Fig. 1. Time course of adhesion and invasion of *C. albicans* to Caco-2 cells. The time points 0.5 h, 2 h and 4 h have been documented using scanning electron microscopy (SEM). The start of invasion can be clearly seen at the time point 4 h, whereas in the preceding panels only adhesion of *C. albicans* to the Caco-2 cell surface is visible. To visualize deep invasive infections, histological staining was performed (Hernandez and Rupp, 2009). Penetration of the cell layer and invasion of the collagen matrix beneath was observable already after 18 h.

of genes in their experiments, consistent with the hypothesis that *C. albicans* reacts differently to distinct epithelia. In these experiments the reference was mid-log growth phase in YPD broth, explaining the large proportion of hyphal-specific genes in comparison to the data we generated. Recently, Park et al. (2009) published a study using basically the same experimental setup and similar conditions we used in our experiments, however, with different epithelial cell lines (FaDu) and endothelial cells (isolated from human umbilical veins). These studies confirmed our finding that changes in expression levels under conditions only different in the growth surface are moderate, up to approximately 4-fold. Also the synthesis of ribosomal proteins was decreased. Typical hyphal-induced genes were not overrepresented in these studies (in fact they were repressed) similar to our observations. Nevertheless, the individual genes detected show only partial overlap with our results. This partial overlap may be due to the different cell lines used, different array types or differences in media as suggested also by Park et al. (2009).

For further analysis we focused on the function of a set of potential cell wall or cell surface proteins identified in our transcriptional approach. Several of the genes potentially involved in cell wall biogenesis were followed up in greater detail by us or in cooperation with partners. For two of the genes *SUN41* and *PRA1* a clear function in host–pathogen interaction could be identified, whereas others like *PGA7* and *PGA23* seem to have a function in cell wall biogenesis in general that may be compensated by other genes as observed by deletion and overexpression studies (unpublished observations).

Sun41p and Pra1p contribute to host–pathogen interaction

SUN41 has been identified as differentially expressed during hyphal development and being regulated by Efg1p (Sohn et al., 2003). In addition, changes of *SUN41* transcript levels between suspension and surface grown cells were detected. However, *SUN41* is expressed under all conditions at moderate to high levels and showed no significant differences in expression levels on different epithelia. The *SUN* gene family has been defined in *Saccharomyces cerevisiae* and comprises a fungus-specific family of proteins, which show high similarities in their C-terminal domains. Genes of this family have been described to be involved in different cellular processes, including DNA replication, aging, mitochondrial biogenesis, and cytokinesis. Furthermore, glucosidase activity has been assigned to this family of proteins based on the characterization of the beta-glycosidase BglBp in *Candida wickerhamii*, homologous to the *SUN* family. In *C. albicans* the *SUN* family comprises two genes, *SUN41* and *SIM1/SUN42*. We demonstrated that *C. albicans* mutants lacking *SUN41* show similar defects as found for *S. cerevisiae*, including defects in cytokinesis. In addition, the *SUN41* mutant showed a higher sensitivity towards the cell wall-disturbing agent Congo red, whereas no enhanced sensitivity was observed in the presence of calcofluor white. This indicates defects in the synthesis of cell wall glucan rather than defects in chitin synthesis. Compared to the wild type, *SUN41* deletion strains exhibited a strong defect in biofilm formation and were unable to form hyphae on solid medium under the conditions tested. In addition, a reduced adherence on a Caco-2 cell monolayer was observed, confirming its importance for colonization of the host. Interestingly, mass-spectrometry analysis of the secretome of SC5314 identified Sun41p as well as Sim1p/Sun42p as secreted proteins of cells growing as blastospores as well as those forming hyphae. This suggests that Sun41p is active at the outer rim of the cell wall (Hiller et al., 2007). In addition, in a parallel study Norice et al. (2007) could show that *SUN41* indeed is required for virulence in a mouse model of systemic infection. They identified *SUN41* in a screen focusing on the analyses of the cell wall through targeted insertional mutagenesis of cell wall-related genes. They also reported defects in biofilm formation and enhanced sensitivity against caspofungin, confirming the defects in glucan synthesis observed by us. In a third approach Firon et al. (2007) identified

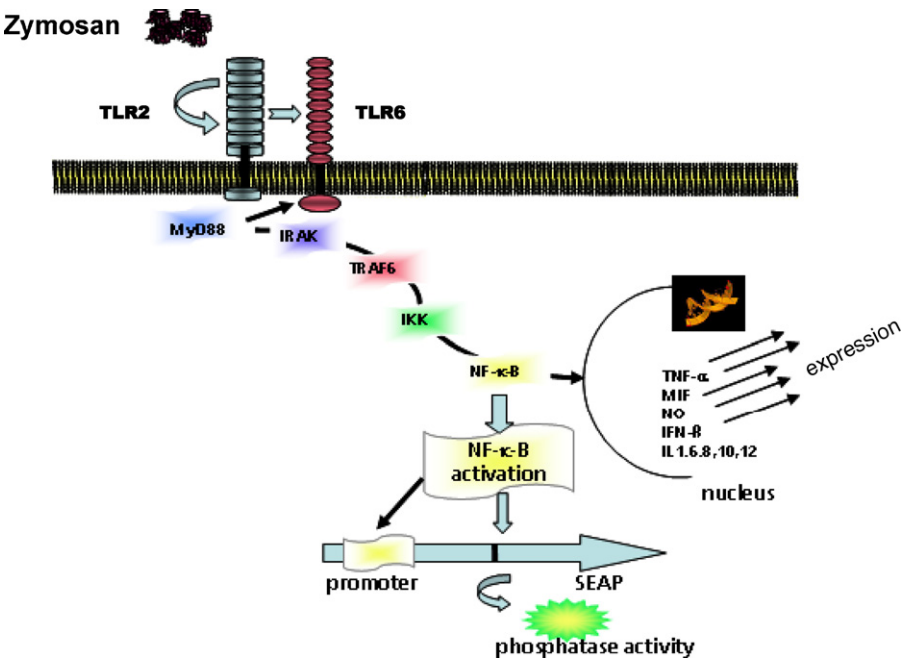

Fig. 2. Schematic representation of the TLR signaling pathway with the link to the NF-κB-dependent reporter gene SEAP (secreted alkaline phosphatase): Stimulation of the receptor combination TLR2/6, e.g. with zymosan, results in initiation of the signaling cascade activating the transcription factor NF-κB. The TLR signaling is visualized by an integrated reporter gene containing an NF-κB-binding site, resulting in NF-κB-mediated activation of the promoter. Expression of the reporter gene SEAP is detected by a simple enzyme substrate reaction resulting in a color change. In immune cells this activation results in the secretion of cytokines like TNF, interleukins and others.

SUN41 in a screen targeted at genes required for biofilm formation. They could show that *SUN41* and *SUN42*/*SIM1*, the only homologue of *SUN41* in *C. albicans*, show synthetic lethality. In conditional mutants deleted for *SUN41* and carrying only one repressable allele of *SUN42*, shutting down the expression of *SUN42* causes lysis of the mother cells during cell separation. These results confirmed the hypothesis that Sun41p together with Sun42p acts as cell wall remodeling enzyme or mediates the function of other glycosidases required for cell–cell separation.

PRA1 was identified as a gene differentially expressed on Caco-2 and A-431 cells (Sohn et al., 2006b). Initially, Pra1p was identified as pH-regulated antigen by immune-screening of a lambda expression library with sera raised against mycelial cell walls of *C. albicans* (Sentandreu et al., 1998). Pra1p was also shown to be highly immunogenic (Viudes et al., 2001), and previously identified as part of the soluble hyphal cell wall fraction of *C. albicans* (Urban et al., 2003). In addition Marcil et al. (2008) showed that *PRA1* is strongly up-regulated after engulfment in macrophages. However, no clear function of the protein was described until recently. Soloviev et al. (2007) showed that Pra1p binds to the integrin $\alpha_M\beta_2$ (CD11b/CD18) using among other approaches affinity chromatography on the purified integrin $\alpha_M\beta_2$. The integrin $\alpha_M\beta_2$ (CD11b/CD18) has been shown to be a major leukocyte receptor involved in *C. albicans* recognition, mediating both adhesive and migratory responses to the fungus. Patients with defects in leukocyte phagocytic functions, like leukocyte adhesion deficiency (LAD-1) lack β_2 integrins and are highly susceptible to fungal infections (Andrews and Sullivan, 2003). Indeed, Pra1p was shown to be required for $\alpha_M\beta_2$-dependent killing of *C. albicans* by PMNs. On the other hand, addition of purified Pra1p protected *C. albicans* from killing by the PMNs. This suggests that the released fraction of Pra1p blocks the integrin $\alpha_M\beta_2$, thereby assisting the fungus in escaping host surveillance.

C. albicans is able to utilize human complement regulators, like Factor H and Factor H-like protein-1 (FHL-1) for immune evasion. Studies by Luo et al. (2009) showed that Pra1p in addition to binding to $\alpha_M\beta_2$ integrins is a novel Factor H- and FHL-1-binding factor. Bound to the surface of *C. albicans*, Pra1p acquires Factor H, FHL-1 and plasminogen, thereby mediating complement evasion, as well as extra-cellular matrix interaction and/or degradation. As a secreted protein, Pra1p enhances complement control in direct vicinity of the yeast and thus generates an additional protective layer which controls host complement attack. This is described in detail by Zipfel et al. (2011). These studies indicate that Pra1p has an important role in host–pathogen interaction in several distinct ways including the modulation of innate immunity as well as immune cells.

These results show that *C. albicans* has developed a highly sophisticated arsenal to modulate the immune system in order to persist and proliferate in a moderate way in various host niches. There will be certainly additional fungal molecules acting as decoys to ensure immune evasion, thereby creating a balance between host and fungus resulting in a commensal stage of colonization.

The host side: PRR-receptors as key sensors for host defense

The last years have seen many novel findings on how the host answers to fungal attack. This includes the identification of pattern recognition receptors (PRRs) required for initiating the first line of defense against fungal invasion (van de Veerdonk et al., 2008). Especially for commensal, facultative pathogens the balance between host and pathogen is crucial (Casadevall and Pirofski, 2000). Therefore, the knowledge about the sensors of our innate immune system, the PRRs and the ligands recognized by them is of high importance for understanding this balance. In order to delineate the individual pathogen-derived ligands recognized by the innate immune system and their receptors, we developed a system in which we are able to investigate isolated PRRs or receptor dimers (hetero- or homo-) without experiencing the highly complex crosstalk between the multitude of receptors and pathways involved in this process (Burger-Kentischer et al., 2010). For this purpose PRRs are stably expressed in an NIH3T3 mouse fibroblast cell line. This cell line does not express to a significant level any of the mouse PRRs tested, providing a system with very

low background. The activation of the individual receptors can be monitored using a simple reporter gene read out (Gfp, or alkaline phosphatase) which is activated by the MyD88-/NF-κB-dependent signal transduction cascade. Inhibitors targeting MyD88 or IκBα block signaling (Fig. 2). We have set up cell lines containing Toll-like receptors (TLR) which have been described to respond to bacterial as well as to fungal cell surface components; CD14/TLR4 responding to LPS, TLR1/2 responding to lipoproteins, and TLR2/6 responding to lipoproteins and zymosan, a cell wall fraction from fungi (mostly prepared from *S. cerevisiae*). These receptor dimers showed a very high sensitivity to bacterial ligands like LPS or synthetic ligands addressing TLR1/2 or TLR2/6 (low pg/ml range). Zymosan also induced activation of TLR2/6 in the low μg range, a concentration range also employed for stimulation of immune cells. An array of additional human PRRs including Dectin 1 and Dectin 2 has been used in this cell-based assay which enables to look at a multitude of individual defined ligands derived from fungal or other pathogens including components of the cell wall or secreted proteins. For Dectin 1 and Dectin 2 we could confirm the activation of both receptors individually by zymosan. In our currently ongoing work we are evaluating additional ligands in this setting with regard to specificity, activation potential and PRR-combinations required. The ability to characterize the individual receptors in an isolated cellular system could open new avenues for understanding the innate immune system and its ability to recognize pathogens.

Conclusion

Within the last decade our knowledge about the mechanisms of host–pathogen interaction of fungal pathogens has increased significantly. This could be realized by a wealth of novel model systems enabling the analysis of host–pathogen interactions in an environment very closely mimicking the host up to patient samples. In combination with genome-wide technologies, like microarrays or proteomics, this resulted in the identification of a whole series of genes directly involved in colonization and invasion of host tissue or modulation of the immune system. Thereby, large strides towards an understanding of the balance between commensalism and pathogenicity have been made. Also on the host side novel tools to unravel specific interactions between molecular patterns of the pathogen and receptors recognizing them are available. These tools, ranging from knock-out mice via combinations of immune cells and epithelial models to reporter cell lines, will further enhance our understanding of pathogenesis of fungi.

In addition, the last few years have seen the development of new technologies like "next generation sequencing" (Margulies et al., 2005), enabling both the rapid and cost-effective generation of pan-genomes as well as meta-transcriptomes of host–pathogen interaction. This technology certainly will advance the fields of genomics and transcriptomics significantly, just by the huge amount of data which can be generated and analyzed in an experimental setup even less complex than it is required for microarray analyses. The use of these technologies further enhances the importance of statistical and mathematical methods of data analysis, so called bioinformatics, for the analysis of the biological process under investigation. Thereby, biology gets more and more close to a mathematically descriptive, quantitative biology in the spirit of Max Delbrück, nowadays summarized as systems biology.

Acknowledgements

This works was supported by the DFG (Ru608/4-1,2,3) within the SPP1160: "Kolonisation und Infektion durch humanpathogene Pilze".

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