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Functional genomics identifies type I interferon pathway as central for host defense against *Candida albicans*

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Abstract

*Candida albicans* is the most common human fungal pathogen causing mucosal and systemic infections. However, human antifungal immunity remains poorly defined. Here, by integrating transcriptional analysis and functional genomics, we identified *Candida*-specific host defense mechanisms in humans. *Candida* induced significant expression of genes from the type I interferon (IFN) pathway in human peripheral blood mononuclear cells. This unexpectedly prominent role of type I IFN pathway in anti-*Candida* host defense was supported by additional evidence. Polymorphisms in type I IFN genes modulated *Candida*- induced cytokine production and were correlated with susceptibility to systemic candidiasis. In *in-vitro* experiments, type I IFNs skewed *Candida*- induced inflammation from a Th17-response toward a Th1-response. Patients with chronic mucocutaneous candidiasis displayed defective expression of genes in the type I IFN pathway. These findings indicate that the type I IFN pathway is a main signature of *Candida*- induced inflammation and plays a crucial role in anti-*Candida* host defense in humans.
Introduction

Candida albicans (C. albicans) is a commensal microorganism that inhabits human skin and mucosa. Candida can be isolated from up to 70% of the general population at any given moment, and it is believed that all individuals have been colonized with Candida at some point. Although it is a commensal organism, C. albicans can also cause mucosal and systemic infections, especially in immunocompromised hosts. Despite the availability of novel classes of antifungal agents (e.g., azoles, echinocandins), mortality due to systemic candidiasis, the fourth most common form of sepsis, reaches 37-44%.

In addition, vaginal and oral candidiasis can also occur in healthy or only mildly immunocompromised individuals, with up to 5% of women in the general population suffering from recurrent vulvovaginal candidiasis.

Despite the introduction of modern antifungal drugs and intensive care facilities, progress in improving the outcome of Candida infections in the last decade has been disappointing. Given this lack of progress, it is currently believed that only adjuvant immunotherapy will be able to further reduce the burden of morbidity and mortality caused by C. albicans infections. Understanding the host defense pathways involved in candidiasis is therefore crucial for identifying novel targets for immunotherapy.

In the present study, we have taken an alternative, unbiased approach to this biological problem. Using a combination of transcriptional analysis and systems biology, we have stimulated human primary cells with the fungus, identifying Candida-specific transcription profiles in human immune cells. While C. albicans induced the transcription of multiple inflammatory gene sets commonly stimulated by other inflammatory stimuli, it also specifically induced several additional gene sets, among which we identified an unexpectedly strong type I IFN signature profile. We confirmed the importance of the type I IFN pathway for anti-Candida host defense in humans through immunological and genetic studies in both healthy volunteers and in patients with systemic candidiasis or suffering from chronic mucocutaneous candidiasis (CMC).

RESULTS

Candida albicans induces a type I interferon response

We measured genome-wide transcriptional profiles in peripheral blood mononuclear cells (PBMCs) from healthy volunteers upon stimulation with C. albicans, as well as with inflammatory stimuli unrelated to fungal pathogens: Escherichia coli-derived lipopolysaccharide (LPS), Borrelia burgdorferi, Mycobacterium tuberculosis (MTB), and RPMI culture medium as a control. Measurements were performed at 4 and 24 hours. 693 transcripts that showed >2-fold higher expression compared to corresponding RPMI stimulation were selected (BH-adjusted P < 0.05). K-means analysis identified profile clusters that indicated shared and specific genes that were differentially expressed in response to each stimulus. We grouped shared genes into signatures representing common early response genes (clusters 1, 6, 14, 27), common late response genes (clusters 2, 3), and common inflammatory genes induced by all stimulations (clusters 9, 21, 25) (Fig. 1a and Supplementary Fig. S1-3).
More importantly, to identify a set of discriminatory features (from the 693 differentially expressed transcripts) that would be most informative of the state of *Candida* stimulation and could distinguish *Candida* stimulation from the other inflammatory stimuli, we utilized information gain (Kullback-Leibler divergence/relative entropy) feature selection, to reduce the dimensionality of the feature space.11,12 We performed 10-fold cross-validation which stratifies the data into 10 random partitions in which each (in turn) is held out for testing and the remainder for training a Bayesian classifier. Class prediction was evaluated by computing the area under the receiver-operating characteristic (ROC) curve (AUROC), which assesses true- and false-positive rates. A 101-transcript (95-gene) *Candida*-response signature (Fig. 1b and Supplementary Table S1) was defined using this supervised learning approach for class prediction, achieving an AUROC of 97.8%, which would indicate an excellent classifier performance with very high discriminative value. Principal component analysis (PCA) of the *Candida*-response signature further demonstrated good class separation on the basis of *Candida* status and stimulation duration (Fig. 1c). As a set, the 101 features collectively identify expression signatures that are informative of *Candida* stimulation across the 4-hour, 24-hour or both time-points that sufficiently distinguish *Candida* from the rest. Enrichment analysis for the 101-transcript *Candida*-response signature revealed a striking over-representation of the interferon (IFN) signaling pathway (p=3.8×10−35) (Fig. 1d and Supplementary Table S2). This set of genes was strongly induced at both 4 hours and 24 hours of *Candida* stimulation and establishes a definitive *Candida*-responsive signature distinct from that observed with LPS, MTB or *Borrelia* stimulation.

**Type I IFN SNPs modulate susceptibility to candidemia**

In order to assess whether the type I IFN genes specifically induced by *C. albicans* in human leukocytes are indeed involved in antifungal host defense, we investigated whether common polymorphisms in a subset of these genes influence susceptibility to systemic *Candida* infections. From a cohort of 217 Caucasian patients with candidemia, well described elsewhere,10,13 we had Immunochip genotyping data available and 11 genes of the 101-transcript (95-gene) dataset were covered by the Immunochip array (Supplementary Table S3). We tested these 11 genes for association by taking immunochip data on 12,228 non-candidemia Caucasian healthy volunteers as controls. Analysis of the loci associated with these 11 genes revealed significant association of genetic variation with susceptibility to systemic candidiasis in four regions: *CCL8* SNP 1kg_17_29697448 (p = 0.00069), *STAT1* SNP rs16833172 (p = 0.0042), *SP110* SNP rs3769845 (p = 0.012) and *PSMB8* SNP rs3198005 (p = 0.01) (Fig. 2a-d) (Supplementary Fig. S1-3, clusters 10, 11, 20, 11 respectively) (Supplementary Table S3). After applying a stringent Bonferroni correction for multiple testing, the association between susceptibility to candidemia and *STAT1* and *CCL8* remained significant. This finding confirms that the *C. albicans*-specific genes we identified in our transcriptional arrays play an important role in host defense against systemic fungal infections. We also attempted to assess whether the polymorphisms in these genes also modified disease severity. However, only 51 patients fulfilled the criteria of severe candidiasis based on clinical scores and persistence of positive blood cultures, and this number was too low for allowing the identification of significant differences between genetic variation and disease severity (disseminated disease, persistent fungemia and mortality at 14 or 30 days) (data not shown).

**Type I IFN SNPs modulate Candida-induced cytokine responses**

In an additional approach to validate the role of the type I IFN pathway for host defense against *Candida*, we isolated PBMCs from an additional group of 74 healthy volunteers and stimulated the cells with the fungus. Genetic variation in 11 type I IFN pathway genes present on the Immunochip was correlated with *C. albicans*-induced release of TNF-α,
IL-1β, IL-8, IL-6, IL-10, IFN-γ and IL-17. In contrast to other microbial stimuli such as LPS and MTB, C. albicans induced higher levels of TNF-α, IL-1β and IL-10 production, and lower levels of IL-8 and IL-6 in human PBMCs (Supplementary Fig. S4). The correlation of cytokine concentrations with genotypes at tag SNPs of Candida-induced IFN pathway genes revealed significant associations at IRF1 and STAT1 regions (Fig. 3a-d) in which IRF1 SNPs were associated with TNFα, (p = 0.0028) and IL-10 levels (p = 0.0024), while STAT1 SNPs were associated with TNF-α (p = 0.001) and IL-6 levels (p = 0.0002). Consistent with the specificity of the type I IFN pathway for Candida stimulation, the association was significant with cytokine levels induced by Candida, but not by LPS or MTB stimulation (Supplementary Fig. S5).

**Type I IFNs skew cytokine responses from Th17 towards Th1**

To investigate the importance of type I IFN signaling in host defense against Candida, PBMCs from healthy individuals were stimulated with heat-killed C. albicans. In line with the transcriptional profile described above (Fig. 1), IFN-β could be detected in cell culture supernatants only after C. albicans stimulation. In contrast, neither live (Fig. 4a), nor heat-killed C. albicans induced any detectable IFN-α production.

We next assessed the role of type I IFN pathways in the induction of cytokines by C. albicans. When PBMCs were stimulated with heat-killed C. albicans in the presence of an antibody against IFN-α/β/ω receptor 1 (IFNAR), IFNγ production was significantly lower compared to PBMCs stimulated in the presence of a control antibody. Blocking of IFNAR did not influence the production of the monocyte-derived cytokines IL-β or TNF-α (Fig. 4b). Conversely, adding IFN-α or IFN-β to Candida-stimulated cells increased IFN-γ production, while significantly decreasing IL-17 and IL-22 production (Fig. 4c). Together these data suggest that type I interferons (in the context of infection, most likely IFN-β) modulate the immune reaction induced by C. albicans, skewing host defense toward a Th1 response.

**STAT1 mutations cause reduced expression of type I IFN genes**

Patients with autosomal dominant CMC have increased susceptibility to mucosal fungal infections and often carry gain-of-function mutations in STAT1, which lead to STAT1 hyperphosphorylation, that in turn likely makes STAT1 unavailable for forming heterodimers with other STAT molecules. In co-cultures of DCs and T-cells from CMC patients, this led to defective Th1 and Th17 responses (Supplementary Fig. S6). In order to validate the role of Candida-specific genes in human antifungal mucosal defense, we assessed the expression of these genes in cells from CMC patients. PBMCs from healthy controls and CMC patients were stimulated with RPMI, E. coli-derived LPS, heat-killed C. albicans, or heat-killed MTB. The expression of type I IFN pathway genes (including IRF5, IRF7, ISG15, IFI44 and IFI44L) that are induced downstream of STAT1-STAT2 showed patterns of defective expression in CMC patients compared to healthy controls, supporting a role of the type I IFN pathway in this immunodeficiency (Fig. 5). The expression of IRF3, IRF9 and IFN-β, three genes in the type I IFN pathway upstream of STAT1-STAT2, was not decreased in cells from CMC patients. In fact, Candida-induced IFN-β expression appeared to be higher in cells from CMC patients, possibly indicating a compensatory feedback mechanism.

In order to investigate the type I IFN pathway in CMC patients in a more systematic way, we also performed RNA sequencing analysis in PBMCs stimulated with C. albicans from both control volunteers and CMC patients. The differential expression of 91 genes in response to C. albicans stimulation was compared between CMC patients and healthy controls (10 genes from the 101-gene signature characteristic for Candida were not
considered: 7 because another isoform was already used for FPKM calculations, and 3 hypothetical proteins without concordant refseq genes). In cells from healthy controls, two genes showed downregulation (LFNG, RGC32), while all other genes were upregulated upon stimulation (Supplementary Fig. S7), validating the previous findings based on expression array analysis. In order to observe differences between patients and controls, the standardized fold change was used to create the respective heatmap (Fig. 6). The patients show a much lower standardized fold change in genes downstream of STAT1, again supporting the role of type 1 IFN pathway in CMC. We found 27 genes, from the set of 91 genes, to be significantly differently regulated upon Candida stimulation in patients compared to controls based on an ANOVA analysis (p-value interaction group and treatment <0.05) (MX1, OAS3, IFIT2, RTP4, XAF1, OAS2, IFI44, IFIH1, IFIT3, ADAR, PRIC285, FAM46A, SAMD9L, EIF2AK2, PARP12, TRIM22, ZBP1, HERC6, RSAD2, IFNG, OAS1, STAT2, DHX58, IRF7, TNFSF10, ISG15, HERC5). To determine the role of these 27 genes we calculated Gene ontology (GO) enrichment scores using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Enriched gene clusters were generated using Functional Annotation Clustering. Gene ontology terms associated with response to type I interferon and viral infections showed significant enrichment (p=1.09×10\(^{-14}\)) (Supplementary Table S4).

**DISCUSSION**

The last decade has witnessed a significant increase in knowledge related to the mechanisms involved in antifungal host defense. However, most of this knowledge is based on in-vitro and animal studies; these studies led to hypotheses to be tested in human systems, leading to a somewhat biased view of human immunity against fungi. In the present study we have used a different approach based exclusively on complementary human studies: transcriptomics analysis in human primary cells, correlation of genetic variability and antifungal immunity in healthy volunteers (functional genomics), case-control association studies in patients with disseminated *C. albicans* infections, and assessment of antifungal immunity in CMC patients.

In our initial approach, we designed a hypotheses-generating set of experiments by assessing the transcriptional profile induced in circulating primary leukocytes by *C. albicans*. Human primary PBMCs were stimulated either with *C. albicans* or with other inflammatory stimuli. Pathway analysis of the genes induced by *Candida* indicated two broad categories: (i) a first set of genes that was strongly induced and shared by both *C. albicans* and other inflammatory stimuli (LPS, MTB, or *B. burgdorferi*), constituted expected pathways involved in inflammation and innate immunity, many of which have important roles in antifungal immunity\(^{18}\), and (ii) a feature set comprising an expression signature of 101 transcripts (95 genes) that distinguishes the *Candida*-specific response and is characterized by a strikingly strong and unanticipated type I IFN response.

Because the identification of the type I IFN pathway as the most prominent *Candida*-specific transcriptional signature was surprising, we initiated a series of studies to confirm this finding. Firstly, we assessed whether genetic polymorphisms in several of the genes identified in the *Candida*-specific dataset influenced susceptibility to systemic *Candida* infections. In a cohort of patients with candidemia that were genotyped using the Immunochip platform (Illumina\(^{14}\)), on which 11 genes of the *Candida*-specific dataset were covered, we identified significant association of genetic variation with susceptibility to candidiasis in four of these genes: *CCL8* locus, *STAT1*, *SP110* locus, and *PSMB8* locus. This finding confirms that genes from the 101 *Candida*-specific dataset influence susceptibility to systemic fungal infections.
Secondly, the role of the type I IFN pathway for the anti-\textit{Candida} immune response was subsequently validated in a study in which PBMCs isolated from a group of healthy volunteers were stimulated with the fungus. Genetic variation in the 11 type I IFN pathway genes present on the Immunochip was correlated with \textit{C. albicans}-induced cytokine production. The correlation of cytokine concentrations with genotypes at tag SNPs of \textit{Candida}-induced IFN pathway genes revealed significant associations at IRF1 and STAT1 regions, both crucial components of type I IFN pathway. We cannot exclude however that the effect of these polymorphisms on TNF-\(\alpha\) production are exerted through type II interferons; IFN-\(\gamma\) is stimulated by type I interferons\(^\text{19}\), and is known to induce TNF-\(\alpha\) production\(^\text{20}\). These data support the hypothesis of the specificity of the type I IFN pathway for \textit{Candida} stimulation, as this association was significant only for cytokine production induced by \textit{Candida}, but not by other stimuli.

Thirdly, we performed classical immunological studies to assess the role of type I IFNs for \textit{Candida}-induced immune responses, and we demonstrated that type I IFNs induced by \textit{Candida} modulate Th1/Th17 cytokine profiles; IFN\(\alpha\) and IFN\(\beta\) increased IFN\(\gamma\) production, while decreasing IL-17 production. Moschen et al previously demonstrated that type I interferons decrease IL-17 production \textit{in vitro} and \textit{in vivo}\(^\text{21}\). TNF-\(\alpha\) production did not seem to be influenced by blocking the IFNAR, suggesting that genetic effects in STAT1 exert their effect on TNF-\(\alpha\) production either through direct intracellular pathways, or indirectly through IFN\(\gamma\). Interestingly \textit{C. albicans} seems to induce only IFN-\(\beta\), rather than IFN-\(\alpha\), consistent with earlier studies in mice\(^\text{22, 23}\).

Finally, using both qPCR and RNA sequencing analysis, we demonstrated that the expression of type I IFN pathway-induced genes was defective in CMC patients harboring a gain of function mutation in STAT1\(^\text{15}\). These mutations lead to increased STAT1 phosphorylation and IFN\(\gamma\) signaling. Liu et al demonstrated increased STAT1 phosphorylation in response to IFN\(\gamma\) stimulation in U3C cells transfected with mutated STAT1\(^\text{16}\). They hypothesize that IFN targeted genes can lead to decreased IL-17 production. However, freshly isolated PBMC from CMC patients do not produce any IFN\(\gamma\) in response to \textit{C. albicans} stimulation\(^\text{15}\). Therefore, it is more likely that the inability of mutated STAT1 to form heterodimers with STAT3 and STAT4 causes defective Th1/Th17 responses\(^\text{17}\), which are likely to represent the main immunological defect in this disease. Additional defects in the expression of type I interferon-induced genes, as shown here, may also contribute to the increased susceptibility to mucosal fungal infections. Whether this is truly caused by a defect in STAT1-STAT2 heterodimerization remains to be demonstrated.

Taken together, these complementary studies represent a compelling body of evidence that the type I IFN pathway is an important component of the immune response during infections with \textit{C. albicans} in humans. Moreover, the data presented here are supported by emerging information from murine studies. For example, Bourgeois \textit{et al} and Biondo \textit{et al} have reported that mouse conventional dendritic cells produce IFN-\(\beta\) upon stimulation with \textit{Candida} spp. and have proposed a role for MyD88, TLR7 and TLR9 in this induction\(^\text{22, 23}\). On the other hand, these results are relatively surprising, as no earlier study has reported a role for type I IFNs in human antifungal host defense. In this respect, primary immunodeficiencies in type I IFN pathway are mainly associated with viral, rather than fungal, infections\(^\text{24}\). Therefore, our data suggest that type I IFN defects determine an increase susceptibility risk for fungal infections, as demonstrated here by the association with candidemia in risk groups, rather than an overt immunodeficiency associated with severe fungal infections.

The precise effect of type I IFNs for anti-\textit{Candida} host defense is still being debated. One study in type I IFNAR knockout mice suggested a deleterious role for this pathway\(^\text{22}\),

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whereas another study reported that IFNAR\(^{-/-}\) mice die from their inability to control fungal growth\(^{23}\). While type I IFNs play an important role in the immune response against viral infections\(^{25}\), they seem to be deleterious in bacterial infections\(^{26}\). In humans with *Candida* infections, the role of type I IFNs is not yet settled, although the defects seen in type I IFN gene expression in CMC patients, the susceptibility to candidemia by STAT1 polymorphisms, and the potentiation of Th1 responses by type I IFNs, all suggest a beneficial effect of the type I IFN pathway in human antifungal host defense.

Although viruses have long been known to induce type I IFN production, other microorganisms have been recently reported to induce this pathway. MTB stimulation induces the expression of IFNB and IFNA1 in human macrophages\(^{27}\) and in murine bone marrow-derived macrophages\(^{28}\); type I IFNs are required for caspase-1 activation during *Francisella tularensis* infection\(^{29}\); and *Listeria monocytogenes* induces the transcription of IFNB in macrophages\(^{30}\). However, the complete transcriptional profile of the type I IFN pathway performed here shows important differences from the type I IFN profile induced by MTB \(^{31}\) and is significantly more pronounced for *Candida* than for MTB (Supplementary Fig. S4). Interestingly, the very strong induction of type I IFN pathway genes by *Candida* may explain the well-known, yet unexplained, beneficial effects of injecting *Candida*-derived extracts for the treatment of warts in patients\(^{32, 33}\).

In summary, this work presents complementary arguments from several studies to propose that the type I IFN pathway plays an important role in the immune responses to the fungal pathogen *C. albicans* in humans. The cross-validation between these approaches supports the value of this systems biology approach, combining transcriptomics with functional genomics and immunological data. Using this approach, we have confirmed the role of pathways known to be involved in antifungal host defense (e.g., common inflammatory pathways and the Th1/Th17 pathways) and we have identified a novel pathway (the type I IFN pathway). It is expected that this knowledge will have an important contribution in devising novel immunotherapeutic strategies for fungal infections, as well as influencing decision-making for vaccine design.

**METHODS**

**Study populations**

For assessing the transcriptome and the cytokine production capacity, blood was collected after written informed consent from healthy volunteers. Adult patients with candidemia were enrolled after informed consent (or waiver as approved by the Institutional Review Board) at the Duke University Hospital (DUMC, Durham, North Carolina, USA) and Radboud University Nijmegen Medical Centre (RUNMC, Nijmegen, The Netherlands). The study was approved by the Institutional Review Boards at each study center, and enrollment occurred between January 2003 and January 2009\(^{34}\). The study was performed in accordance with the declaration of Helsinki. In addition, blood was collected from two patients with CMC (on two different occasions) and eight healthy controls in order to investigate the induction of IFN genes by *Candida*. After informed consent was given, blood was collected by venipuncture into 10 ml EDTA syringes (Monoject, s-Hertogenbosch, The Netherlands).

**PBMC isolation**

Separation and stimulation of peripheral blood mononuclear cells (PBMCs) was performed as described previously\(^{35}\). Briefly, the PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice in saline and suspended
in culture medium. The cells were counted in a hemocytometer and their number was adjusted to 5·10^6/mL.

**Cell stimulation**

For the transcriptome analysis, cells were stimulated for 4 or 24 hours with RPMI, heat-killed *Borrelia burgdorferi* [36], *Candida albicans* (UC 820) (1·10^6/mL) [37], *Mycobacterium tuberculosis* (1 μg/mL) or *E. coli*-derived LPS (10 ng/mL). In order to correlate cytokine production to SNPs in the type I IFN pathway, 5×10^5 PBMCs from healthy volunteers were stimulated with heat-killed *C. albicans* (1·10^9/mL) for 24 hours. In order to investigate the effect of type I IFNs on cytokine production, 5×10^5 PBMCs were stimulated with heat-killed *C. albicans* (1·10^9/mL) in the absence or presence of IFN-α 2b (1·10^5 U/mL)(IntronA, SP Labo N.V., Heist-op-den-berg, Belgium), IFN-β (10 ng/mL), or anti-IFNAR (100 ng/mL)(21385-1, PBL Interferon Source, Piscataway, USA). To assess the development of Th1 and Th17 cells, a total of 1×10^6 mononuclear cells was added to 96-wells flat-bottom plates (Greiner). Subsequently, the monocytes within the PBMC pool were differentiated towards dendritic cells (DCs) by addition of IL-4 (50 ng/ml, R&D Systems), GM-CSF (50 ng/ml, R&D Systems) and 10% human serum for seven days. After every second day, half of the medium was replaced by fresh DC differentiation medium. At day seven of the culture, cells were stimulated with 1×10^6 *Candida* conidia for another six days.

**Transcriptome analysis**

Global gene expression was profiled using Illumina Human HT-12 Expression BeadChip according to manufacturer’s instructions. Image analysis, bead-level processing and quantile normalization of array data were performed using the Illumina LIMS platform, BeadStudio. A gene-wise linear model was fitted to the data and was performed in R programming language (http://www.r-project.org/), utilizing functions from Linear Models for Microarray Data [38]. For each probe, a moderated t-statistic (with standard errors moderated across genes) was computed using a Bayesian model. The associated p values were adjusted to control the false discovery rate in multiple testing, using the Benjamini and Hochberg’s (BH) method [39]. For each transcript, Signal-to-Noise ratios (SNR) were computed for each infection or stimuli group relative to the untreated (RPMI) control group. The expression SNR profiles of transcripts identified as differentially expressed (BH-adjusted p<0.05 and fold-change>2x) were clustered using the K-means algorithm [40]. We performed feature (attribute) selection [41, 42] on differentially expressed transcripts using Information Gain (Kullback-Leibler divergence or relative entropy) to identify discriminatory features or transcripts that are most informative of the state of infection or stimulation. The accuracy of class prediction using a Java implementation of a naïve Bayes classifier [42, 43] incorporating these features was evaluated using 10-fold cross-validation.

**Cytokine measurements**

The concentration of IL-1β, IL-17, IL-22, TNF-α (R&D Systems, Abingdon, UK), IL-6, IL-8, IL-10, IFN-γ (Sanquin, Amsterdam, the Netherlands), and IFN-α, IFN-β (PBL Interferon Source, Piscataway, USA) was measured in cell culture supernatants using enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer.

**SNP selection**

DNA was isolated using the Gentra Pure Gene Blood kit (Quiagen, Venlo, The Netherlands), according to the protocol of the manufacturer. Illumina Immunochip is a custom designed High-Density array to densely genotype 186 loci associated with 12 different autoimmune or inflammatory diseases reaching genome-wide association study

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significance threshold (P<5×10^{-8}). The candidemia cohort and volunteers DNA samples were genotyped on this Immunochip Illumina platform. Only SNPs in 11 of the 101-gene dataset identified earlier were studied, as the other genes were not represented on the Immunochip. We selected tag-SNPs (Supplementary Table S3) at each region that are located within ±250 kb of the locus by employing Haplovie44 and we excluded SNPs with r² ≥ 0.8 from association analysis.

**Statistical analysis**

Analyses were performed using PLINKv1.0745, 46. We performed Multidimensional scaling (MDS) analysis to reveal genetic background of cases and controls in Candidemia cohort (Supplementary Fig. S8). The association between tag-SNPs and Candidemia susceptibility was tested by logistic regression with the first four components from MDS analysis as covariates. The correlation between cytokine levels and genotypes was tested by Wilcoxon rank sum test using R. P-values less than 0.05 was considered significant.

**Real-time PCR**

Two million freshly isolated PBMCs were incubated with various stimuli as described above. After 24 hours of incubation at 37°C, total RNA was extracted in 800 μl of TRIzol reagent (Invitrogen, Breda, The Netherlands). Isolated RNA was reverse-transcribed into complementary DNA using oligo(dT) primers and MMLV reverse transcriptase. PCR was performed using a 7300 Real-Time PCR system (Applied Biosystems, Lennik, Belgium). Primer sequences for human IFN-β: sense: 5′-ATG-ACC-AAG-TGT-CTC-CTC-C-3′ and antisense: 5′-GGA-ATC-CAA-GCA-AGT-TGT-AGC-TC-3′; IRF9: sense 5′-GAT-ACA-GCT-ACC-ATG-TTC-TC-3′ and antisense: 5′-TGA-TAC-ACC-TTG-TAG-GGC-TCA-3′; IFI7: sense: 5′-GCT-GGA-CGT-GAC-CAT-CAT-GTA-3′ and antisense: 5′-GGG-CCG-TAT-AGG-AAC-GTC-G-3′; ISG15: sense: 5′-CGC-AGA-TCA-CCC-AGA-AGA-TCG-3′ and antisense: 5′-TTT-GCA-CTG-ATT-GTG-CC-3′; IFI44: sense: 5′-ATG-GCA-CTG-ACA-ATG-CTT-3′ and antisense: 5′-GCA-GGA-CTC-ATG-CCA-3′; IFI44L: sense: 5′-GGC-CTG-AGA-TGG-GTC-AAC-G-3′ and antisense: 5′-GCC-TCC-GTT-GTA-3′. B2M was used as a reference gene, for which the primers were: 5-ATG-AGT-ATG-CCT-GCC-GTG-TG-3 (forward) and 5-CCA-AAT-GCG-GCA-TCT-TCA-AAC-3 (reverse). PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of PCR reaction at 95°C for 15 seconds, and 60°C for 1 minute.

**RNA sequencing**

RNA sequencing experiments were performed on 4 hour unstimulated and C. albicans-stimulated PBMC from three healthy controls and two CMC patients. Total RNA was isolated as described above. The RNA integrity (RNA integrity score ≥7) was measured on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA) using an Agilent RNA 6000 Pico Chip according to the manufacturer’s instructions. Enrichment of mRNA was done using the MicroPoly(A)Purist Kit (Ambion, Life Technologies, Foster City, USA) according to manufacturer’s instructions, starting with 5μg of total RNA and using two rounds of Oligo(dT) selection. Whole transcriptome library preparation was performed using the SOLiD Total RNA-Seq Kit (STaR Kit, Life Technologies) and the corresponding low input amount protocol (version July 2011, Life Technologies). In brief, 25ng high quality poly(A) RNA was fragmented by RNase III digestion, cleaned up using the PureLink RNA Micro Kit (Invitrogen, Life Technologies) and checked for quantity and size-distribution on an Agilent 2100 Bioanalyzer. RNA adapters were hybridized and ligated to the fragmented...
RNA, followed by reverse transcription. After purification and size-selection of cDNA using Agencourt AMPure XP Reagent beads, the cDNA was amplified and used as input for the SOLiD system bead preparation using the SOLiD Easy bead system (E120 scale). Paired-end sequencing (50+25 bases) was performed on one flowcell of a 5500XL sequencer (Life Technologies) For each sample deep sequencing was performed resulting in a sequencing depth ranging from 75.6 million to 105.5 million paired-end reads. Paired-end colorspace reads were mapped against the human genome (hg19) using Tophat (version 1.4.1)\(^47\) To increase mapping performance a GTF-file containing the exon boundries of all known RefSeq genes (25,024 genes) was supplied to Tophat and the insert size was set to 110 bases. For all genes FPKM (Fragments per Kilobase per Million mapped reads) values were calculated using Cufflinks (version 2.0.0)\(^48\). Further statistical analyses was done using Partek® Genomic Suite software (version 6.6, build 6.12.0109, Partek Inc., St. Louis, USA). Fold changes and p-values were calculated for each gene by performing a multifactorial Analysis of Variance (ANOVA) on log2 (with an offset of 1.0) transformed FPKM expression values. Factors included in the ANOVA model were group (patient or control), treatment (stimulation status), individual (data from same individual), and the interaction between group and treatment. Fold changes for the heatmaps were calculated for each individual by dividing the FPKM expression values of the C. albicans stimulated sample by the unstimulated (RPMI) sample. Fold changes with values below 1 were replaced by the negative of the inverse. A gene list containing 101 differentially expressed transcripts known from the expression array was used to select genes for further downstream analysis. Of this list, 10 transcripts were not considered; 7 because another isoform was already used for FPKM calculations, and 3 hypothetical proteins without concordant refseq genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES


Figure 1. Transcriptional responses to Candida

(a) K-means clustering identifies shared and specific gene signatures in response to infection or stimulation. For example, clusters 21 and 25 indicate common inflammatory genes (details in Supplementary Fig. S2) that are significantly differentially induced genes (Benjamini-Hochberg-adjusted-p<0.05, fold-change>2) in response to Borrelia burgdorferi, Candida albicans, E.coli-derived LPS, and Mycobacterium tuberculosis (M.tb), compared to RPMI stimulations (Untreated), while clusters 7, 11 and 20 identify Candida-dominant response genes. Signal-to-noise ratio relative to untreated is shown in the heatmap. (b) Using feature selection, naïve Bayes classifier and 10-fold cross-validation, a 101-transcript feature set was identified from 693 differentially expressed transcripts shown in (a), which distinguishes Candida from the other stimulants and an area under the ROC (Receiver operating characteristic) curve of 97.8%, indicating a high discriminative value. The expression profile (Z-transformed) of this feature set is displayed. (c) Principal component analysis (PCA) of the Candida-response signature. Data from each individual sample is plotted along the three main principal components (PC1, PC2, PC3). The three main principal components account for 45% of the total variance in gene expression. Samples from the various stimulations and infections are color coded as follows: Candida albicans (2h: bright red; 24 hr: dark red), Borrelia burgdorferi (Green), E.coli-derived LPS (Blue), Mycobacterium tuberculosis (M.tb) (Purple) and untreated (Yellow). (d) Enrichment analysis (MetaCore pathways) of the 101-transcript feature set reveals a striking enrichment of the interferon (IFN) signaling pathway. Supplementary Table S2 displays all the signaling/biological processes shown and their enrichment p-values.
Figure 2. SNPs of type I IFN responsive genes are associated with susceptibility to Candidemia
Regional association plots, at (a) STAT1 region (b) CCL8/CCL13 region (c) SP110 region and (d) PSMB8 region, showing chromosome position in the x-axis and the –log of logistic P values for each SNP in the y-axis. Blue diamond indicates the top P value; while orange and yellow diamonds indicates r² ranges of 0.6 to 0.8 and 0.4 to 0.59, respectively with the top associated SNP. SNP names shown are as in dbSNP130 where available. Otherwise, the Illumina Immunochip manifest name is shown. For example 1kg_17_29697448 indicates the location of SNP on chromosome 17 at position 29697448 (hg18).
Figure 3. SNPs of type I IFN responsive genes are associated with *Candida*-induced cytokine levels

Correlation plots showing the association between genotypes in the x-axis and cytokines levels in the y-axis. Association between (a) TNF-alpha levels and genotypes at IRF1 SNP rs2548997, (b) IL-10 levels and genotypes at IRF1 SNP rs1124211, (c) TNF-alpha and genotype at STAT1 SNP rs2280234 and (d) IL-6 levels and genotypes at STAT1 SNP rs41511150.
Figure 4. Type I IFNs skew cytokine production from a Th1 towards a Th17 response

(a) $5 \times 10^5$ PBMC in a volume of 200 $\mu$L, were stimulated for seven days, in the presence of 10% human pooled serum, with live Candida albicans ($1 \times 10^6$/mL), in duplo. IFN-α and IFN-β were measured in cell culture supernatants using ELISA. (b) $5 \times 10^5$ PBMC in a volume of 200 $\mu$L, were stimulated for one and two days, with heat-killed Candida albicans ($1 \times 10^6$/mL), in the absence of presence of anti-IFN-α/βR2, in duplo. IFN-γ, IL-1β and TNF-α were measured in cell culture supernatants using ELISA. (c) $5 \times 10^5$ PBMC in a volume of 200 $\mu$L, were stimulated for two and seven days, with heat-killed Candida albicans ($1 \times 10^6$/mL), in the absence of presence of IFN-α or IFN-β, in duplo. When cells were cultured for seven days, this was done in the presence of 10% human pooled serum. IFN-γ, IL-17 and IL-22 were measured in cell culture supernatants using ELISA. (a,b,c) After stimulation, supernatants were pooled and stored at −20°C until further assayed. Data were analyzed using the Wilcoxon signed rank test. Data are presented as mean + SEM.
Figure 5. Expression of type I IFN pathway genes in patients with a STAT1 mutation
Reduced expression of type I IFN pathway genes in patients with a STAT1 mutation (n = 2, with patients measured twice), and healthy controls (n = 8). 5 · 10^5 PBMC in a volume of 200 μL, were stimulated for 24 hours, with RPMI, E. coli-derived LPS (10 ng/mL), heat-killed Candida albicans (1 · 10^6/mL), or heat-killed Mycobacterium tuberculosis (1 μg/mL). mRNA was isolated from cell lysates using TRIzol. Transcription of IFN-β, IRF3, IRF9, IRF5, IRF7, ISG15, IFI44 and IFI44L was measured using qPCR. Data are presented as mean + SEM.
Figure 6. Heatmap of response of CMC patients and controls to *Candida albicans in vitro* stimulation

In this analysis the standardized fold change gene expression in stimulated vs. unstimulated cells was calculated. The scale of this heatmap is given as standardized log2 fold change with a range from −1.8 (blue) to +1.8 (red).