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The RIG-I-like helicase receptor MDA5 (*IFIH1*) is involved in the host defense against *Candida* infections

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Abstract

The induction of host defense against *Candida* species is initiated by recognition of the fungi by pattern recognition receptors and activation of downstream pathways that produce inflammatory mediators essential for infection clearance. In the present study, we present complementary evidence based on transcriptome analysis, genetics, and immunological studies in knockout mice and humans, that the cytosolic RIG-I-like receptor MDA5 (*IFIH1*) has an important role in the host defense against *C. albicans*. Firstly, *IFIH1* expression in macrophages is specifically induced by invasive *C. albicans* hyphae, and patients suffering from chronic mucocutaneous candidiasis (CMC) express lower levels of MDA5 than healthy controls. Secondly, there is a strong association between missense variants in the *IFIH1* gene (rs1990760 and rs3747517) and susceptibility to systemic *Candida* infections. Thirdly, cells from Mda5 knockout mice and human PBMCs with different *IFIH1* genotypes display an altered cytokine response to *C. albicans*. These data strongly suggest that MDA5 is involved in immune responses to *Candida* infection. As a receptor for viral RNA, MDA5 receptor until now has been linked to antiviral host defense, but

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these novel studies show unexpected effects in antifungal immunity as well. Future studies are warranted to explore the potential of MDA5 as novel target for immunotherapeutic strategies.

Keywords

Candida albicans; host defense; candidemia; genetic susceptibility; MDA5; *IFIH1*

Introduction

Candida species are one of the most common human fungal pathogens. They inhabit skin, mucosa, and the gastrointestinal tract. Normally, *Candida* colonization does not lead to disease in healthy individuals; however, this peaceful cohabitation can drastically change when the host immune system is compromised. Oropharyngeal and vaginal *Candida* infections are relatively commonly present in the population, while systemic candidiasis is the fourth most common form of bloodstream infections in the US. It is often life-threatening due to invasion of deep tissues and organs, with mortality rates reaching up to 40% [1-4].

Recent progress has partly elucidated the environmental and genetic risk factors that contribute to the causes and severity of *Candida* infections [5, 6]. Immunosuppressive medication, treatment with antibiotics, and prolonged hospitalization in intensive care units, are some of the most important risk factors [7, 8]. Several monogenic disorders that result in primary immunodeficiencies increase the susceptibility to *Candida* infection, as demonstrated for mutations in *CARD9* and *STAT1* [9-11]. Common genetic variants, for example in pattern recognition receptors (e.g. *Dectin-1* and *TLR1*) and interleukins (e.g. *IL-4*, *IL-10* and *IL-12B*), also increase the risk of infection by affecting *Candida* recognition and cytokine signaling [6, 12]. It is likely that combinations of these and other common genetic variants in multiple genes underlie the complex patterns of susceptibility to different forms of disease caused by *Candida* spp.

The discovery of genetic factors contributing to infection susceptibility has improved our understanding of the molecular pathways involved in the immune response against *Candida*. However, identifying pathway components that are suitable targets for novel immunotherapeutic approaches requires more insight. Recently, Smeekens *et al.* discovered that the type I interferon (IFN) pathway plays a central role in host defense against *C. albicans* [13]. In the present work, we demonstrate that MDA5 (*IFIHI*), a RIG-I-like receptor, until now described as a receptor of viral RNA that induces a signaling pathway leading to the production of type I interferons, is directly involved in the inflammatory response against *Candida* infections in humans. To this end, we present complementary evidence based on genetics, gene expression, and functional immunological studies in knockout mice and in healthy humans, as well as in patients suffering from systemic candidiasis or chronic mucocutaneous candidiasis (CMC). This is the first time that a receptor of the RIG-I-like helicase family has been shown to be involved in the antifungal immune response.

Materials and Methods

Macrophage transcriptome analysis after stimulation with wild-type and *hgc1*-defective *Candida albicans*

CD14+ monocytes were derived from healthy volunteers and differentiated into macrophages using M-CSF for 7 days. 200000 monocytes per well remained unstimulated or were stimulated for 4 or 24 hours with either wild-type *C. albicans* (UC820) or a *HGC1* null mutant, which is unable to form hyphae. Global gene expression was then profiled as previously described [13]. Gene expression levels in the various conditions were compared to unstimulated macrophages. We identified genes that showed significant differential expression after Benjamini-Hochberg (BH)-correction ($P < 0.05$ and > 2-fold change in expression) in at least one of the conditions (i.e. stimulation with wild-type or *HGC1* null *Candida*, for 4h or 24h, compared to unstimulated control). From this set, we selected the 62 genes that are exclusively induced after stimulation with wild-type *C. albicans* for 24 hours. These genes were analyzed for enrichment in KEGG pathways [49] using the DAVID suite [50], with all human genes as background.

Candidemia and control cohorts

In this study we included 227 unrelated adult Caucasian patients suffering from candidemia (described in detail in [6]). Patients were enrolled after giving written informed consent at the Duke University Hospital (DUMC, Durham, North Carolina, USA) as well as the Radboud University Medical Centre (Radboudumc, Nijmegen, The Netherlands). Patient enrollment took place after confirmation of at least one positive blood culture for a *Candida* species. The study was approved by the Institutional Review Boards of both medical centres, the Institutional Review Board of Duke University (CR4_Pro00006427) in North Carolina (USA) and the 'Commissie Mensgebonden Onderzoek Arnhem-Nijmegen' (2001/198) in Nijmegen (The Netherlands). The study was performed in accordance with the international guidelines of the declaration of Helsinki (year 2000) of the World Medical Association adopted by the World Medical Assembly. Enrollment took place between January 2003 and January 2009.

A control cohort of 176 Caucasians was employed. This cohort consists of non-infected (candidiasis free) matched patients from the same medical centres as the patient cohort. Controls were recruited consecutively from the same hospital wards/services as infected patients during the study period, with a similar balance of medical, surgical, and oncology patients in case and control groups.

Genotyping and quality control

The case and control individuals of the candidemia cohort were genotyped on the Illumina Immunochip SNP array platform, which contains ~200,000 SNPs focused on genomic regions known to be involved in immune-mediated diseases [16]. DNA was isolated from whole blood using the Gentra Pure Gene Blood kit (Qiagen, Venlo, The Netherlands), according to the protocol of the manufacturer. Only samples with a SNP call rate above 90% were included. We applied quality control filters to exclude SNPs with: (i) a genotype call rate of less than 90%, (ii) strong deviation from Hardy-Weinberg equilibrium in control

samples (Hardy-Weinberg exact test $P \leq 1 \times 10^{-3}$), and (iii) significant differences in missingness between cases and controls (Fisher's exact $P < 1 \times 10^{-2}$). As our cohort does not have the power to detect associations with rare variants, we only included SNPs with a minor allele frequency (MAF) of greater than 5% in cases and controls together. 843 SNPs in the *FAP-IFIH1-GCA-KCNH7* LD region were tested in the case-control association analysis.

Genetic analyses

Linkage disequilibrium (LD) measures were calculated using Haploview version 4.2 [51] for both the candidemia cohort (LD patterns are based on genotypes of control individuals only) and common SNPs (MAF > 5%) in the CEU population (Utah Residents (CEPH) with Northern and Western European ancestry) in HapMap 3, release 2 [52]. The 405 kb *FAP-IFIH1-GCA-KCNH7* LD region on chromosome 2 (hg18 coordinates: 162,720 kb – 163125 kb) was defined based on the LD patterns in the larger genomic region (**Figure S1**). It includes the complete *FAP* gene and part of the *KCNH7* gene.

Associations between SNPs and susceptibility to candidemia were assessed using both genotypic and allelic tests. Genotypic association was calculated using the Fisher's exact test, asking whether candidemia cases and controls have significantly different genotype count distributions (H_0: genotype counts are the same). We corrected for testing multiple SNPs using the Benjamini-Hochberg procedure. Similarly, allelic association was calculated using the Fisher's exact test, asking whether candidemia cases and controls have significantly different allele count distributions (H_0: allele counts are the same). Odds ratios (OR, with 95% confidence intervals) are reported for the allelic association tests and represent the odds of disease for individuals carrying the non-risk allele versus the risk allele. Quality filtering and genetic analyses were performed using PLINK v1.07 [53] and custom R scripts. Regional association plots were created using code adapted from http://www.broadinstitute.org/diabetes/scandinavs/figures.html.

Expression analysis of PBMCs

To assess the expression levels of genes in the *FAP-IFIH1-GCA-KCNH7* LD region, blood was collected from healthy volunteers after written informed consent. Isolated peripheral blood mononuclear cells (PBMCs) were obtained using density centrifugation described previously [43]. 0.5×10^6 isolated PBMCs remained unstimulated (RPMI), or were stimulated with either 1×10^6/ml heat killed *Borrelia burgdorferi* [54], 1×10^6/ml heat killed *Candida albicans* (UC820) [55], 10 ng/ml *Escherichia coli*-derived lipopolysaccharide (LPS), or 1×10^7/ml sonicated *Mycobacterium tuberculosis* (MTB) (Hv37Rv) for either 4 or 24 hours. Gene expression was profiled using Illumina Human HT-12 expression BeadChip following the protocol of the manufacturer [13]. Data generation and processing was performed as described elsewhere [13]. Additionally, we measured gene expression in PBMCs from two patients suffering from chronic mucocutaneous candidiasis (CMC) as well as healthy controls. Both CMC patients carried the *STAT1* mutation (Arg274Trp) described earlier [10]. Cells were stimulated with *C. albicans* for 4 hours and samples subsequently underwent RNA sequencing. RNA isolation as well as enrichment, library preparation and data processing are described elsewhere [13].

PBMC stimulation experiments

After obtaining written informed consent, PBMCs were isolated from blood of healthy volunteers using density centrifugation as described elsewhere [43]. 0.5×10^6 cells were plated in 96 wells round bottom plates and subsequently stimulated with either heat killed *C. albicans* yeast or hyphae (both UC820, 1×10^6/ml, heat-killed at 100°C for 1 hour) for 24 hours (IL-10) or 7 days (IL-17 and IFN-γ). Additionally, PBMCs were stimulated with *Mycobacterium tuberculosis* (MTB) (1μg/μl) for 24h. Supernatants were collected and measured for IL-10 and IFN-γ (Sanquin, Amsterdam, The Netherlands), and IL-17 cytokines (R&D Systems, Abingdon, UK) according to the instructions of the manufacturers.

Mda5 knockout mice studies

Mda5$^{-/-}$ mice on C57BL/6J background that have been backcrossed at least ten times (kindly provided by dr. M. Colonna) were housed in the facilities of the University Hospital Bonn, Germany. C57BL/6J wild-type mice were purchased from Charles River Breeding Laboratories. All *in vivo* mice protocols were approved by the internal animal care committee of the University Hospital Bonn and were performed according to national and European regulations.

Splenocytes were isolated from wild-type and *Mda5*$^{-/-}$ mice and stimulated with RPMI, Poly I:C, and heat-killed *C. albicans* yeasts or hyphae for 24 hours. Supernatants were stored at −20°C before cytokine measurement by ELISA. For qPCR, splenocytes from both Mda5 knockout mice and B6 control mice were stimulated for 24 hours with heat-killed *C. albicans* hyphae. RNA was isolated according to the TRIzol® isolation protocol supplied by the manufacturer (Life Technologies).

Results

Candida germination induces expression of RLR pathway components in macrophages

Candida albicans is a dimorphic fungus that exists either in a colonizing yeast form, or as an invasive filamentous form (hyphae). In a first set of experiments we aimed to identify the specific transcription profile induced in human macrophages by *C. albicans* hyphae. To this end, we profiled gene expression in macrophages stimulated with wild-type and *HGC1* null (yeast-locked) strains of *C. albicans* for either 4 or 24 hours, the latter time point being required for fungal germination into hyphae (**Figure 1**). *HGC1* is required for the yeast-to-hyphae transition and *HGC1* null *Candida* fails to form hyphae, thus remaining in the colonizing yeast form [14]. We identified 62 genes exhibiting significant differential expression specifically in macrophages stimulated with *Candida* hyphae for 24h, but not for 4h, nor in macrophages stimulated with yeast-locked *Candida* (Benjamini-Hochberg (BH)-corrected $P < 0.05$ and > 2-fold change in expression, compared to unstimulated macrophages, **Figure 1A**). Many of these genes are involved in interferon (IFN) signaling, consistent with a previous study uncovering an important role for type I interferons in the response to *Candida* [13]. Interestingly, four of the genes induced by *Candida* hyphae stimulation (*IFIH1*, *ISG15*, *IL8*, and *TRIM25*) are key components of the RIG-I-like

receptor (RLR) signaling pathway, which is significantly more than expected for a random set of genes ($P = 4.3 \times 10^{-3}$, 11.5-fold enrichment, **Table 1** and **Figure 1B**).

RIG-I-like receptors are well-known intracellular receptors of viral RNA leading to the production of type I IFNs and proinflammatory cytokines [15]. *IFIH1* (interferon induced with helicase C domain 1), with its protein product known as MDA5 (melanoma differentiation-associated protein 5), is a receptor of long double-stranded RNA (dsRNA), which constitutes one branch of the RLR pathway (**Figure 1B**). *ISG15* and *TRIM25* are involved in the RIG-I branch of that pathway, which is activated by shorter, 5' triphosphate-containing dsRNA. The two branches of the pathway converge at the signaling adaptor MAVS (also known as IPS-1). Thus, the invasive form of *Candida* induces expression of components of two branches of the virus-recognition RLR pathway in macrophages.

Genetic variation linked to *IFIH1* modulates susceptibility to candidemia

To validate this unexpected role for components of the virus recognition RLR pathway, *IFIH1*, *ISG15*, *IL8*, and *TRIM25*, in invasive *Candida* infection, we investigated whether genetic variation linked to these genes correlates with susceptibility to candidemia. For this, a cohort of 227 Caucasian patients with candidemia and 176 matched controls (described in detail in [6]) was genotyped using the Immunochip single-nucleotide polymorphism (SNP) array, which contains ~200,000 SNPs focused on genomic regions known to be involved in immune-mediated diseases [16]. We checked for the presence of SNPs associated with the four RLR pathway components on the array and tested the loci for association with candidemia. *ISG15* and *TRIM25* were not covered, and genetic variation linked to *IL8* did not reveal any association.

In contrast, analysis of 64 SNPs associated with *IFIH1* revealed strong allelic and genotypic association with susceptibility to candidemia (**Figure 2A** and **Table 2**). The *IFIH1* locus is present in a 405 kb genomic region on chromosome 2 with low recombination rates (**Figure 2A**) and accompanying strong linkage disequilibrium (LD) in both the candidemia cohort (**Figure 2B** and **Figure S1D**) and the HapMap CEU population (**Figures S1A-C**), as also seen in other studies [17-19]. Besides *IFIH1*, the LD region contains the genes *FAP* (fibroblast activation protein), *GCA* (grancalcin), and part of *KCNH7* (potassium voltage-gated channel subfamily H member 7). Of the 64 SNPs in the *FAP-IFIH1-GCA-KCNH7* LD region, 15 have genotype count distributions that differ significantly between cases and controls after applying Benjamini-Hochberg multiple testing correction (BH-corrected genotypic $P < 0.05$, **Table S1**). The significant SNPs are distributed mainly across the central part of the LD region (**Figure 2A**). Indeed, the association with candidemia does not extend beyond the LD region (**Figure S2**). An intergenic SNP between *GCA* and *KCNH7* shows the strongest association with candidemia in the *FAP-IFIH1-GCAKCNH7* LD region (rs984971, genotypic $P = 2.2 \times 10^{-5}$, allelic $P = 2.2 \times 10^{-4}$, odds of disease 0.43 – 0.77, **Table 2**). Interestingly, although the Immunochip covers missense coding variants in all four genes, only *IFIH1* harbors significant missense SNPs (rs1990760 – Ala946Thr, and rs3747517 – His843Arg, which are also in strong LD with each other; HapMap CEU: D' = 1, r2 = 0.42 – candidemia cohort: D' = 1, r2 = 0.55).

The strong LD across the region makes that our genetic data cannot definitively assert which SNP(s), and hence which gene(s), are responsible for the observed association with candidemia. However, it has previously been shown that rs1990760 and rs3747517, among other *IFIH1*-linked SNPs, are quantitative trait loci (QTLs) to *IFIH1* expression, with the candidemia risk alleles correlating with higher *IFIH1* expression in PBMCs [18, 20]. Thus, the presence of (missense) SNPs in the *IFIH1* locus showing significant association with the infection, together with the observed *IFIH1* upregulation in macrophages responding to *Candida* hyphae, strongly suggest the involvement of *IFIH1* in the host defense against candidemia.

IFIH1 is strongly upregulated upon *Candida* stimulation of PBMCs, while *FAP, GCA,* and *KCNH7* are not

To provide additional evidence regarding which genes in the *FAP-IFIH1-GCA-KCNH7* LD region are important for the host response to *Candida*, we measured gene expression in peripheral blood mononuclear cells (PBMCs) from healthy volunteers in the presence of *C. albicans* and various other stimuli for 4 or 24 hours. 4h stimulation with bacterial stimuli such as *Borrelia burgdorferi* and *Escherichia coli*-derived lipopolysaccharide (LPS) resulted in increased expression of *IFIH1*, but not of the other LD region genes (**Figure 3A**). Importantly, stimulation with *C. albicans* resulted in an even higher *IFIH1* expression. Of the other genes, only *GCA* was also induced by *C. albicans*, but this expression was negligible compared to *IFIH1*. Similar trends were apparent after 24h of stimulation: *C. albicans* induced high expression of *IFIH1* (though lower than at 4h), and moderate expression of *GCA*, while *FAP* and *KCNH7* show no effect (**Figure 3A**). Heat-killed *Mycobacterium tuberculosis* also induced expression of *IFIH1* at 24h, although to a lesser extent than *C. albicans*.

To improve support for a likely involvement of *IFIH1* induction in antifungal host defense, we compared the expression patterns of *IFIH1* in cells from three healthy individuals with cells isolated from two patients suffering from chronic mucocutaneous candidiasis (CMC) due to a deleterious *STAT1* mutation [10]. For this, PBMCs were isolated and stimulated with *C. albicans* for 4 hours, after which gene expression was measured using RNA sequencing. CMC patients expressed significantly lower levels of *IFIH1* after stimulation with *C. albicans* than healthy individuals ($P = 0.04$, Welch-corrected t test, **Figure 3B**). The differences in expression were largely specific for *IFIH1*, although we also observed minor differences in the expression of *GCA* ($P = 0.05$, Welch-corrected t test, **Figure 3B**). Together, the observations in healthy individuals and CMC patients indicate that expression of *IFIH1*, and not expression of the other genes in the *FAP-IFIH1-GCA-KCNH7* LD region, is specifically induced by stimulation with *C. albicans* but not by bacterial stimuli. This suggests that *IFIH1* plays an important role in the antifungal host defense.

Genetic variants in *IFIH1* are associated with an altered cytokine profile in response to *Candida*

To investigate the functional consequences of genetic variants associated with *IFIH1* that predispose individuals to candidemia (rs984971, rs1990760 and rs3747517, **Table 2**), we correlated the genotypes of these SNPs with *in vitro* cytokines levels upon *Candida*

stimulation in healthy volunteers. IL-10, IFN-γ, and IL-17 levels produced by PBMCs were measured after stimulation with either *C. albicans* yeasts or hyphae. Interestingly, we observed a trend towards an increased capacity to release the proinflammatory cytokines IFN-γ and IL-17 in cells isolated from individuals homozygous for the risk allele for both *IFIH1* missense polymorphisms (TT for rs1990760; CC for rs3747517) (**Figure 4**). In contrast, levels of the anti-inflammatory IL-10 tended to be lower in individuals carrying the risk allele for these SNPs (**Figure 4**). This decrease was consistent between stimulation with the *C. albicans* yeast and hyphal forms. The top SNP associated with candidemia (intergenic rs984971) did not reveal the same general trends as the two missense SNPs (**Figure S3**). Furthermore, stimulation with *Mycobacterium tuberculosis* did not reveal clear correlations between cytokine levels and *IFIH1* missense SNP genotypes (**Figure S4**), strengthening the previous observation that the involvement of *IFIH1* under the stimuli studied here is specific for *Candida*. Thus, genetic variation in *IFIH1* influences anti-*Candida* cytokine profiles *in vitro*.

Missense SNPs could affect MDA5 protein function

We next sought to gain insight into the possible consequences of having alternative alleles at the *IFIH1* missense SNPs on MDA5 protein function. MDA5 arose from a duplication of the MDA5/LGP2 gene in the ancestor of jawed vertebrates [21]. Analysis of MDA5 orthologs in 59 jawed vertebrates reveals that both amino acids of rs1990760 (Ala946Thr) are common (alanine: 36/59=61%, threonine: 10/59=17%), while for rs3747517 (His843Arg) the type sequence of human is the only sequence that encodes a histidine (similar data in [22]). The occurrence of alternative alleles at both SNPs suggests that both lead to a functional protein, although there may be functional differences.

Residue 946 is part of an intrinsically disordered loop [23] in the C-terminal domain (CTD) of MDA5. The equivalent loop is rigid in RIG-I, and this differential flexibility contributes to the different RNA binding preferences: displacement of the loop upon dsRNA binding allows MDA5 to recognizes long dsRNA, while the loop in RIG-I specifically caps shorter dsRNA [24]. The human MDA5 crystal structure has an arginine at position 843, which interacts with the negatively charged RNA backbone (**Figure S5**). Histidine would weaken this electrostatic interaction because it is less often positively charged at physiological pH than arginine. Furthermore, position 843 is close to the interface likely involved in interactions between MDA5 monomers (**Figure S5**) [24]. The formation of MDA5 filaments along the RNA is critical for downstream activation of MAVS [25]. Indeed, mutation of nearby residues 841 and 842 disrupts filament formation and signaling [24]. Thus, the Ala946Thr and His843Arg substitutions could alter dsRNA binding selectivity and affinity, and the latter might also affect signaling activity. Furthermore, as noted above, these and other SNPs also correlate with *IFIH1* gene expression.

Mda5 knockout mice have reduced cytokine production in response to *C. albicans*

To provide an additional argument for the role of MDA5 in the anti-*Candida* response, we stimulated splenocytes from Mda5 knockout mice and B6 control mice (C57BL/6J) with *C. albicans* yeasts or hyphae. Quantitative PCR analysis showed a defective production of interferon β induced by *Candida* in the cells isolated from mice deficient in Mda5 compared

to controls, comparable to the positive control stimulus poly I:C (**Figure 5**). Similarly, the IL-6 and IL-10 cytokine responses were also lower under *Candida* stimulation of cells from Mda5 knockout mice, and these differences were more pronounced when cells were stimulated with hyphae compared to the yeast form (**Figure S6**).

Discussion

In the present study we propose that the pattern recognition receptor MDA5, which belongs to the RIG-I-like receptor (RLR) family and is known for its role in antiviral immunity, is also involved in antifungal host defense. Through a combined approach involving transcriptomics, genetic susceptibility studies and immunological validation, these experiments demonstrate that MDA5 modulates cytokine production induced in human leukocytes by *C. albicans*, while genetic variants in the *IFIH1* gene that encodes MDA5 influence susceptibility to disseminated candidiasis. Based on these data and the known role of MDA5 in interferon production, it is most likely that this effect is mediated through induction of type I interferons (IFN).

MDA5 is composed of two N-terminal CARD domains, responsible for the signaling activity, coupled to a central DExD/H box helicase domain and a C-terminal domain (CTD), which together recognize the long dsRNA often associated with replicating viruses [15]. RNA binding causes MDA5 to interact with MAVS in a CARD-dependent manner, leading to downstream signaling and subsequent activation of transcription factors such as IRF3, IRF7, and NF-κB. These factors induce the expression of type I interferons that are a mainstay of antiviral host defense [26]. Surprisingly, transcriptome analysis aiming to identify the immunological programs induced in human macrophages by *Candida* identified the MDA5/RIG-I signaling pathway as the top target. *C. albicans* is a dimorphic fungus, and germination from yeasts to hyphae is a central process for the invasion of tissues, often at the level of the mucosa. We therefore aimed to identify the mechanisms through which human macrophages respond specifically to the germination process. We approached this by stimulating the macrophages either with wild-type *C. albicans* that germinates during the 24h culture period, or with the *hgc1*-defective strain that is unable to form hyphae. Pathway analysis identified the MDA5 pathway as essential among the macrophage genes that were specifically induced by hyphae. The hypothesis that MDA5 is important for host defense against *Candida* is strengthened by the observation that MDA5 induction was significantly reduced in cells isolated from patients suffering from chronic mucocutaneous candidiasis.

Additional evidence that MDA5 is indeed important for human antifungal defense was provided by the assessment of genetic variation predisposing to candidemia. In a cohort of candidemia patients we found a strong association between the disease and genetic variation in the linkage disequilibrium (LD) region on chromosome 2 that contains *IFIH1*. While genetic variation in *IFIH1* has previously been shown to influence susceptibility to several autoimmune diseases such as type I diabetes, Graves' disease, and multiple sclerosis [18-20, 27-32], this is the first report of polymorphisms in *IFIH1* linked to a fungal infection. These data are in line with recent studies showing that polymorphisms in other pattern recognition receptors such as *TLRs* [6, 33-35], or components of the IFN pathway such as *STAT1* or *IRF1* [13], also influence susceptibility to systemic fungal infections.

It is important to point out that the candidemia-associated LD region contains several genes: *FAP* (fibroblast activation protein), *IFIH1* (interferon induced with helicase C domain 1), *GCA* (grancalcin) and *KCNH7* (potassium voltage-gated channel subfamily H member 7). *IFIH1* and grancalcin are the strongest *a priori* candidates for causing the susceptibility to candidemia because, in contrast to *FAP* and *KCNH7*, these genes have known functions in immunity. Grancalcin is abundant in macrophages and neutrophils, particularly those recovered from sites of bacterial infection [36], and is thought to mediate leukocyte adhesion and migration [37]. To obtain direct evidence for a role of genes in the LD region in candidemia susceptibility, we assessed their gene expression in PBMCs stimulated with *Candida*. *IFIH1* was strongly induced and *GCA* to lesser extent, while the other genes did not show any expression changes. *IFIH1* and *GCA* are divergently transcribed neighboring genes with ~25kb separating their transcription start sites. As such neighboring genes tend to be co-expressed more often than random genes [38, 39], the moderate upregulation of *GCA* in response to *Candida* stimulation could be a by-effect of the strong induction of *IFIH1*, although the genes are still relatively far apart.Importantly, grancalcin-deficient ($Gca^{-/-}$, homozygous mutant) mice do not have an increased susceptibility to candidiasis compared to wild-type mice [40], which strongly argues against an important role for *GCA* in the immune response against *C. albicans*. Therefore, we concluded that genetic variants acting at *IFIH1* are the most likely cause of the association of the *FAP-IFIH1-GCA-KCNH7* LD region with candidemia.

The candidemia risk alleles of the *IFIH1*-linked SNPs identified in our study have previously been shown to lead to higher expression of *IFIH1* in PBMCs [18, 20]. Furthermore, our protein structure analyses indicate a possibly stronger RNA binding by MDA5 in the presence of an arginine at position 843, which is encoded by the risk allele C of rs3747517. To gain insight into the downstream immunological effects of *IFIH1* variants, we measured cytokine levels produced by PBMCs from donors with different genotypes for the two *IFIH1* missense SNPs (rs1990760 and rs3747517). These data indicate that cells from individuals bearing the candidemia risk alleles produce more proinflammatory cytokines (IFN-γ and IL-17) and less anti-inflammatory IL-10 in response to *C. albicans* yeast and hyphal forms than cells bearing the protective alleles.

These observations bring into discussion the nature of the involvement of MDA5 in the host defense against *Candida*. MDA5 activates the RLR pathway leading to the production of type I interferons during viral infections [26]. A similar biological activity during *Candida* stimulation was shown by our data from Mda5 knockout mouse splenocytes, which displayed a decreased capacity to induce IFN-β compared to control cells. A role for type I IFNs in antifungal immunity has been recently proposed [13], and MDA5 is likely the receptor that is at least partially responsible for the type I IFN induction during *C. albicans* infection.

A recent study showed that rare *IFIH1* variants causing the type I interferon-mediated pathology of the Aicardi-Goutières syndrome are gain of function mutations that lead to stronger RNA binding, and thereby increase baseline and RNA-induced IFN signaling [41]. Therefore, other mutations leading to inherently increased expression or activity of MDA5, such as those associated with candidemia as identified in this study, are likely to increase

IFN production in a similar manner. Aberrant production of type I IFNs in turn might be cause imbalances in the immune response that are reflected in the observed alterations in the levels of other cytokines.

The apparent deleterious effect of MDA5 hyperactivity on the anti-*Candida* host defense is consistent with observations that type I IFNs could be harmful for this response as well: mice defective in type I interferon receptors (Ifnar1$^{-/-}$ mice) are actually more resistant to systemic *Candida* infections [42]. This is also in line with our findings that PBMCs with the candidemia risk genotype in *IFIH1* tend to release more inflammatory cytokines. The hypothesis that MDA5 has a negative effect on the anti-*Candida* immune response has been proven by a very recent elegant study demonstrating that Mda5$^{-/-}$ mice are more resistant to disseminated candidiasis (Malireddi and Kanneganti, personal communication).

It is currently unclear which ligands cause activation of MDA5 in *Candida* infection. *Candida* is mainly recognized by cell surface pattern recognition receptors such as TLRs and C-type lectin receptors (CLRs), after which the fungus is internalized and subsequently digested in the phagolysosome [43, 44]. It is conceivable that, during this process, *Candida*-derived structures may leak from these organelles and enter the cytoplasm, a process earlier described for the recognition of mycobacterial peptidoglycans by the cytoplasmic receptor NOD2 [45-47]. Interestingly, a recent study has suggested that NOD2 is also important for the recognition of *Candida* chitin [48]. Nevertheless, we currently have no experimental evidence to support that either the wild-type form or a variant form of MDA5 has other ligands than the described RNAs, and future studies should investigate this aspect.

In conclusion, this study demonstrates that the viral receptor MDA5 has an important role in modulating the innate immune response against the fungal pathogen *C. albicans*. Genetic variation in this gene is associated with candidemia, which is likely mediated by a shift in cytokine responses. Future research should aim to shed light on the exact mechanisms through which MDA5 participates in the immune response against the fungus. Nevertheless, the possible deleterious effects of MDA5-dependent stimulation during systemic candidiasis shown by our data suggest its potential usefulness as a novel therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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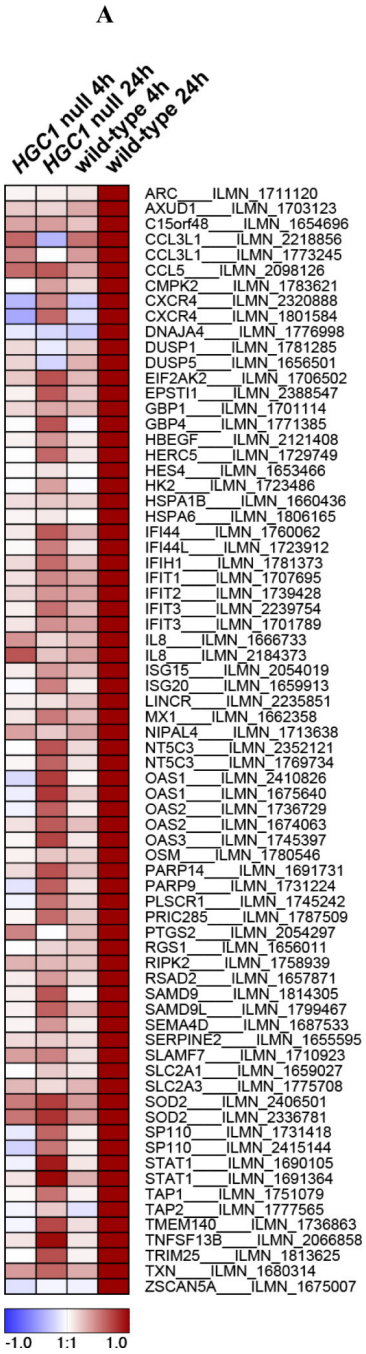
References

1. Gudlaugsson O, Gillespie S, Lee K, et al. Attributable mortality of nosocomial candidemia, revisited. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. Nov 1; 2003 37(9):1172–7. [PubMed: 14557960]

2. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. Aug 1; 2004 39(3):309–17. [PubMed: 15306996]
3. Miller LG, Hajjeh RA, Edwards JE Jr. Estimating the cost of nosocomial candidemia in the united states. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. Apr 1.2001 32(7):1110.
4. Zaoutis TE, Argon J, Chu J, Berlin JA, Walsh TJ, Feudtner C. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. Nov 1; 2005 41(9):1232–9. [PubMed: 16206095]
5. Smeekens SP, van de Veerdonk FL, Kullberg BJ, Netea MG. Genetic susceptibility to Candida infections. EMBO molecular medicine. Jun; 2013 5(6):805–13. [PubMed: 23629947]
6. Plantinga TS, Johnson MD, Scott WK, et al. Toll-like receptor 1 polymorphisms increase susceptibility to candidemia. The Journal of infectious diseases. Mar 15; 2012 205(6):934–43. [PubMed: 22301633]
7. Kullberg BJ, Verweij PE, Akova M, et al. European expert opinion on the management of invasive candidiasis in adults. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. Sep; 2011 17(Suppl 5):1–12.
8. Guery BP, Arendrup MC, Auzinger G, et al. Management of invasive candidiasis and candidemia in adult non-neutropenic intensive care unit patients: Part II. Treatment. Intensive care medicine. Feb; 2009 35(2):206–14. [PubMed: 18972100]
9. Glocker EO, Hennigs A, Nabavi M, et al. A homozygous CARD9 mutation in a family with susceptibility to fungal infections. The New England journal of medicine. Oct 29; 2009 361(18): 1727–35. [PubMed: 19864672]
10. van de Veerdonk FL, Plantinga TS, Hoischen A, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. The New England journal of medicine. Jul 7; 2011 365(1):54–61. [PubMed: 21714643]
11. Liu L, Okada S, Kong XF, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. The Journal of experimental medicine. Aug 1; 2011 208(8):1635–48. [PubMed: 21727188]
12. Babula O, Lazdane G, Kroica J, Linhares IM, Ledger WJ, Witkin SS. Frequency of interleukin-4 (IL-4) -589 gene polymorphism and vaginal concentrations of IL-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. May 1; 2005 40(9):1258–62. [PubMed: 15825027]
13. Smeekens SP, Ng A, Kumar V, et al. Functional genomics identifies type I interferon pathway as central for host defense against Candida albicans. Nature communications. 2013; 4:1342.
14. Zheng X, Wang Y, Wang Y. Hgc1, a novel hypha-specific G1 cyclin-related protein regulates Candida albicans hyphal morphogenesis. The EMBO journal. Apr 21; 2004 23(8):1845–56. [PubMed: 15071502]
15. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. Mar 19; 2010 140(6): 805–20. [PubMed: 20303872]
16. Trynka G, Hunt KA, Bockett NA, et al. Dense genotyping identifies and localizes multiple common and rare variant association signals in celiac disease. Nature genetics. Dec; 2011 43(12): 1193–201. [PubMed: 22057235]
17. Qu HQ, Marchand L, Grabs R, Polychronakos C. The association between the IFIH1 locus and type 1 diabetes. Diabetologia. Mar; 2008 51(3):473–5. [PubMed: 18071670]
18. Liu S, Wang H, Jin Y, et al. IFIH1 polymorphisms are significantly associated with type 1 diabetes and IFIH1 gene expression in peripheral blood mononuclear cells. Human molecular genetics. Jan 15; 2009 18(2):358–65. [PubMed: 18927125]
19. Smyth DJ, Cooper JD, Bailey R, et al. A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. Nature genetics. Jun; 2006 38(6):617–9. [PubMed: 16699517]

20. Downes K, Pekalski M, Angus KL, et al. Reduced expression of IFIH1 is protective for type 1 diabetes. PloS one. 2010; 5(9)
21. Mukherjee K, Korithoski B, Kolaczkowski B. Ancient origins of vertebrate-specific innate antiviral immunity. Molecular biology and evolution. Jan; 2014 31(1):140–53. [PubMed: 24109602]
22. Molineros JE, Maiti AK, Sun C, et al. Admixture mapping in lupus identifies multiple functional variants within IFIH1 associated with apoptosis, inflammation, and autoantibody production. PLoS genetics. 2013; 9(2):e1003222. [PubMed: 23441136]
23. van der Lee R, Buljan M, Lang B, et al. Classification of Intrinsically Disordered Regions and Proteins. Chemical reviews. Apr 29.2014
24. Wu B, Peisley A, Richards C, et al. Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. Cell. Jan 17; 2013 152(1-2):276–89. [PubMed: 23273991]
25. Hou F, Sun L, Zheng H, Skaug B, Jiang QX, Chen ZJ. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell. Aug 5; 2011 146(3): 448–61. [PubMed: 21782231]
26. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature. May 4; 2006 441(7089):101–5. [PubMed: 16625202]
27. Chen G, Zhou D, Zhang Z, et al. Genetic variants in IFIH1 play opposite roles in the pathogenesis of psoriasis and chronic periodontitis. International journal of immunogenetics. Apr; 2012 39(2): 137–43. [PubMed: 22152027]
28. Yang H, Wang Z, Xu K, et al. IFIH1 gene polymorphisms in type 1 diabetes: genetic association analysis and genotype-phenotype correlation in Chinese Han population. Autoimmunity. May; 2012 45(3):226–32. [PubMed: 22053898]
29. Sutherland A, Davies J, Owen CJ, et al. Genomic polymorphism at the interferon-induced helicase (IFIH1) locus contributes to Graves' disease susceptibility. The Journal of clinical endocrinology and metabolism. Aug; 2007 92(8):3338–41. [PubMed: 17535987]
30. Cen H, Wang W, Leng RX, et al. Association of IFIH1 rs1990760 polymorphism with susceptibility to autoimmune diseases: a meta-analysis. Autoimmunity. Nov; 2013 46(7):455–62. [PubMed: 23734776]
31. Martinez A, Santiago JL, Cenit MC, et al. IFIH1-GCA-KCNH7 locus: influence on multiple sclerosis risk. European journal of human genetics : EJHG. Jul; 2008 16(7):861–4. [PubMed: 18285833]
32. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science. Apr 17; 2009 324(5925):387–9. [PubMed: 19264985]
33. van der Graaf C, Kullberg BJ, Joosten L, et al. Functional consequences of the Asp299Gly Toll-like receptor-4 polymorphism. Cytokine. Jun 7; 2005 30(5):264–8. [PubMed: 15927851]
34. van der Graaf CA, Netea MG, Drenth IP, te Morsche RH, van der Meer JW, Kullberg BJ. Candida-specific interferon-gamma deficiency and toll-like receptor polymorphisms in patients with chronic mucocutaneous candidiasis. The Netherlands journal of medicine. Nov; 2003 61(11):365–9. [PubMed: 14768719]
35. Van der Graaf CA, Netea MG, Morre SA, et al. Toll-like receptor 4 Asp299Gly/Thr399Ile polymorphisms are a risk factor for Candida bloodstream infection. European cytokine network. Mar; 2006 17(1):29–34. [PubMed: 16613760]
36. Liu F, Shinomiya H, Kirikae T, Hirata H, Asano Y. C haracterization of murine grancalcin specifically expressed in leukocytes and its possible role in host defense against bacterial infection. Bioscience, biotechnology, and biochemistry. Apr; 2004 68(4):894–902.
37. Lollike K, Johnsen AH, Durussel I, Borregaard N, Cox JA. Biochemical characterization of the penta-EF-hand protein grancalcin and identification of L-plastin as a binding partner. The Journal of biological chemistry. May 25; 2001 276(21):17762–9. [PubMed: 11279160]
38. Trinklein ND, Aldred SF, Hartman SJ, Schroeder DI, Otillar RP, Myers RM. An abundance of bidirectional promoters in the human genome. Genome research. Jan; 2004 14(1):62–6. [PubMed: 14707170]

39. Michalak P. Coexpression, coregulation, and cofunctionality of neighboring genes in eukaryotic genomes. Genomics. Mar; 2008 91(3):243–8. [PubMed: 18082363]
40. Roes J, Choi BK, Power D, Xu P, Segal AW. Granulocyte function in grancalcin-deficient mice. Molecular and cellular biology. Feb; 2003 23(3):826–30. [PubMed: 12529388]
41. Rice GI, del Toro Duany Y, Jenkinson EM, et al. Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling. Nature genetics. May; 2014 46(5):503–9. [PubMed: 24686847]
42. Majer O, Bourgeois C, Zwolanek F, et al. Type I interferons promote fatal immunopathology by regulating inflammatory monocytes and neutrophils during Candida infections. PLoS pathogens. 2012; 8(7):e1002811. [PubMed: 22911155]
43. Netea MG, Gow NA, Munro CA, et al. Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. The Journal of clinical investigation. Jun; 2006 116(6):1642–50. [PubMed: 16710478]
44. Djeu JY. Role of tumor necrosis factor and colony-stimulating factors in phagocyte function against Candida albicans. Diagnostic microbiology and infectious disease. Sep-Oct;1990 13(5): 383–6. [PubMed: 2126496]
45. Ferwerda G, Girardin SE, Kullberg BJ, et al. NOD2 and toll-like receptors are nonredundant recognition systems of Mycobacterium tuberculosis. PLoS pathogens. Nov; 2005 1(3):279–85. [PubMed: 16322770]
46. Rahman A, Sobia P, Gupta N, Kaer LV, Das G. Mycobacterium tuberculosis subverts the TLR-2-MyD88 pathway to facilitate its translocation into the cytosol. PloS one. 2014; 9(1):e86886. [PubMed: 24475192]
47. Stamm LM, Morisaki JH, Gao LY, et al. Mycobacterium marinum escapes from phagosomes and is propelled by actin-based motility. The Journal of experimental medicine. Nov 3; 2003 198(9): 1361–8. [PubMed: 14597736]
48. Wagener J, Malireddi RK, Lenardon MD, et al. Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. PLoS pathogens. Apr.2014 10(4):e1004050. [PubMed: 24722226]
49. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research. Jan 1; 2000 28(1):27–30. [PubMed: 10592173]
50. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009; 4(1):44–57. [PubMed: 19131956]
51. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. Jan 15; 2005 21(2):263–5. [PubMed: 15297300]
52. International HapMap Consortium. Altshuler DM, Gibbs RA, et al. Integrating common and rare genetic variation in diverse human populations. Nature. Sep 2; 2010 467(7311):52–8. [PubMed: 20811451]
53. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. American journal of human genetics. Sep; 2007 81(3):559–75. [PubMed: 17701901]
54. Oosting M, Ter Hofstede H, Sturm P, et al. TLR1/TLR2 heterodimers play an important role in the recognition of Borrelia spirochetes. PloS one. 2011; 6(10):e25998. [PubMed: 21998742]
55. Lehrer RI, Cline MJ. Interaction of Candida albicans with human leukocytes and serum. Journal of bacteriology. Jun; 1969 98(3):996–1004. [PubMed: 4182532]
56. International HapMap Consortium. Frazer KA, Ballinger DG, et al. A second generation human haplotype map of over 3.1 million SNPs. Nature. Oct 18; 2007 449(7164):851–61. [PubMed: 17943122]
57. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. Science. Jun 21; 2002 296(5576):2225–9. [PubMed: 12029063]



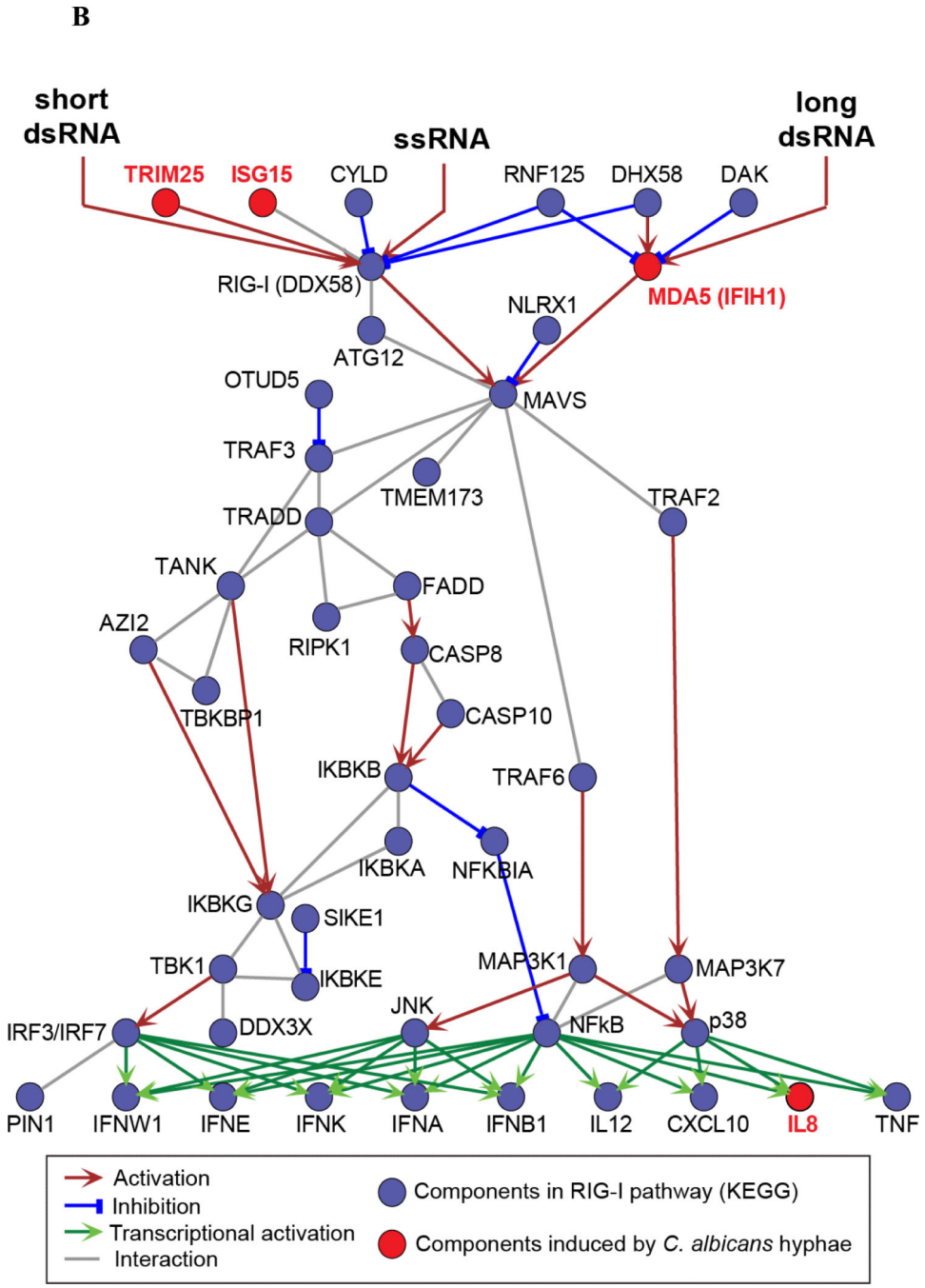


Figure 1. Transcriptional changes in macrophages stimulated with *Candida albicans*

(**A**) The heatmap shows differential gene expression after 4h or 24h stimulation of human macrophages with yeast-locked *HGC1* null *Candida albicans* (which are unable to form hyphae), or wild-type invasive *Candida albicans* (that can form hyphae), compared to expression levels in unstimulated macrophages (control). 62 genes exhibited a significant change in expression level (Benjamini-Hochberg-corrected $P < 0.05$ and > 2-fold change in expression) specifically after 24h stimulation with wild-type *Candida*, during which germination into hyphae takes place. Signal-to-noise ratio scaled to the maximum absolute

deviation is shown for each probe corresponding to the 62 differentially expressed genes. (**B**) *Candida albicans* hyphae-induced genes, *IFIH1*, *TRIM25*, *ISG15* and *IL8* (indicated in red), are key components of the RIG-I-like receptor (RLR) signaling pathway. These genes represent both the MDA5 (*IFIH1*) and RIG-I (*ISG15* and *TRIM25*) branches, as well as inflammatory cytokines that are produced by activation of the pathway (*IL8*). Figure based on the KEGG map of the RLR pathway [49].

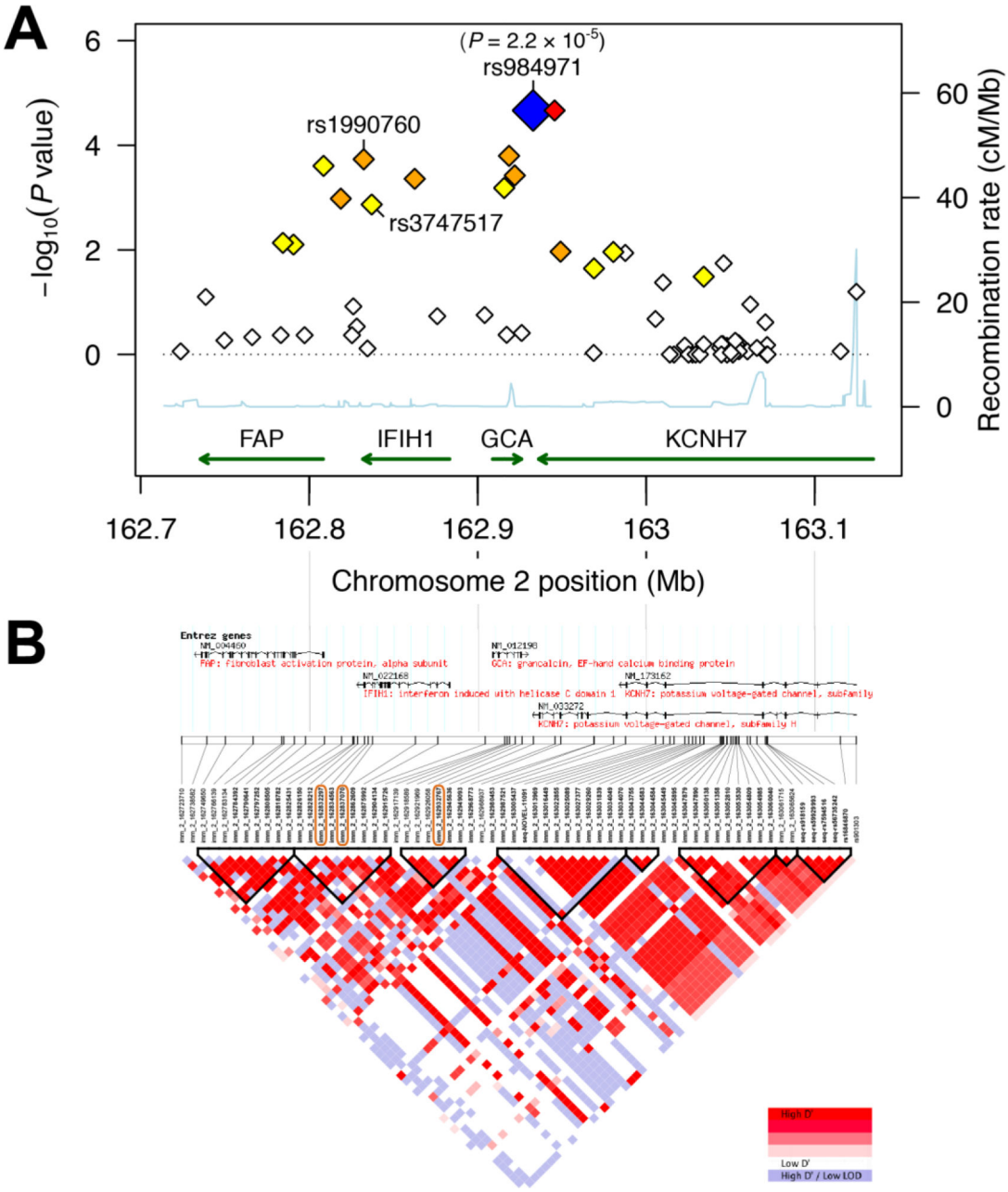


Figure 2. (A) Regional association plot and (B) linkage disequilibrium (LD) map for the *FAP-IFIH1-GCA-KCNH7* LD region on chromosome 2

(**A**) 64 SNPs with MAF > 5% in 403 Caucasian individuals of the candidemia cohort (cases and controls together) were assessed for genotypic association with candidemia. The resulting - $\log_{10}$(genotypic *P* values) (left *y*-axis) are plotted as a function of genomic coordinates (hg18, *x*-axis). The blue diamond highlights the most significant SNP with its *P* value (rs984971). rs1990760 and rs3747517 are the only two significant missense SNPs; both are in the coding region of *IFIH1*. Recombination rates, estimated from the CEU, YRI and JPT+CHB HapMap populations (HapMap 2, Release 22) [56], are plotted to reflect the local LD structure (right *y*-axis, cyan line). SNPs are colored according to the degree of LD

with the most significant SNP, rs984971 (R-squared, calculated across the controls in the candidemia cohort; from strong to weak LD - red: r2≥0.8; orange: 0.5≤r2<0.8; yellow: 0.2≤ r2<0.5; white: r2<0.2). Genes with their direction of transcription are shown at the bottom; *KCNH7* is only partly in this region. (**B**) LD patterns across the 405 kb *FAP-IFIH1-GCA-KCNH7* LD region are calculated based on genotypes of control individuals in the candidemia cohort, measured using the Immunochip SNP array. The intersections of the diagonals between pairs of SNPs are colored according to the degree of LD, which is calculated as D' and LOD: SNPs with D' values between 0 and 1 and with LOD ≥ 2 are colored from white to red. Haplotype blocks (triangles with bold black borders) are regions where at least 95% of SNPs are in strong LD, defined by high D' values [57]. Chromosome 2 coordinates (hg18) and Entrez genes are shown at the top. Orange boxes around SNP identifiers indicate the top SNP and two *IFIH1* m SNPs significantly associated with susceptibility to candidemia (see **Table 2**). The corresponding R-squared LD map for the candidemia cohort is depicted in **Figure S1D**. See **Figures S1A-C** for R-squared and D'/LOD LD maps calculated based on the HapMap CEU population.

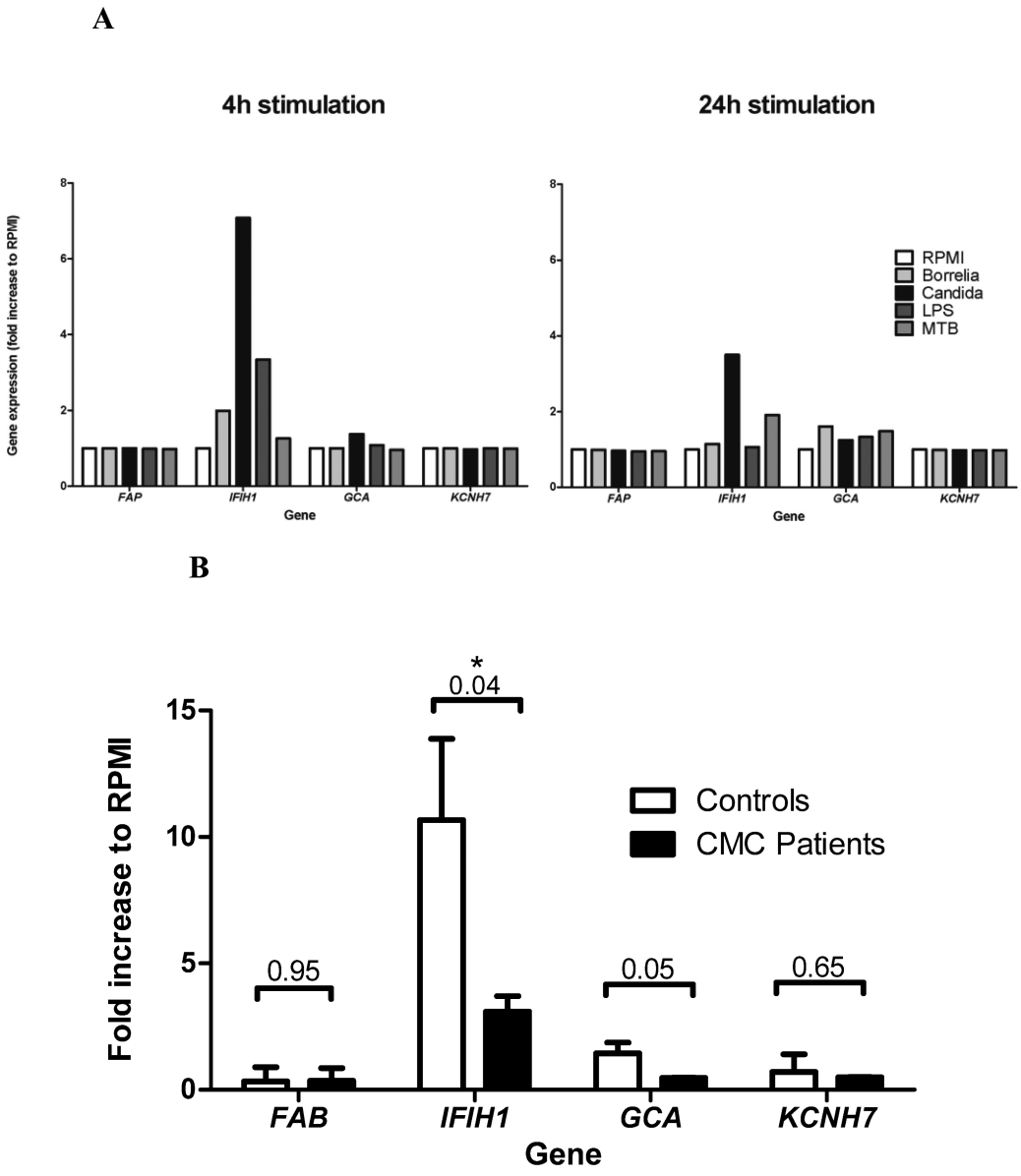


Figure 3. Transcriptional response of genes in the *FAP-IFIH1-GCA-KCNH7* LD region to various microbial stimuli

(**A**) Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were stimulated for either 4 or 24 hours with *Borrelia burgdorferi, Candida albicans*, *Escherichia coli*-derived lipopolysaccharide (LPS), or *Mycobacterium tuberculosis* (MTB). Gene expression was measured using microarrays and normalized to the control RPMI condition (untreated). (**B**) Gene expression (Mean ± SD) in PBMCs of healthy controls (n=3) and patients suffering from chronic mucocutaneous candidiasis (CMC) (n=2) were stimulated with *C. albicans* for 4 hours. *P* values were calculated using the Welch-corrected t test.

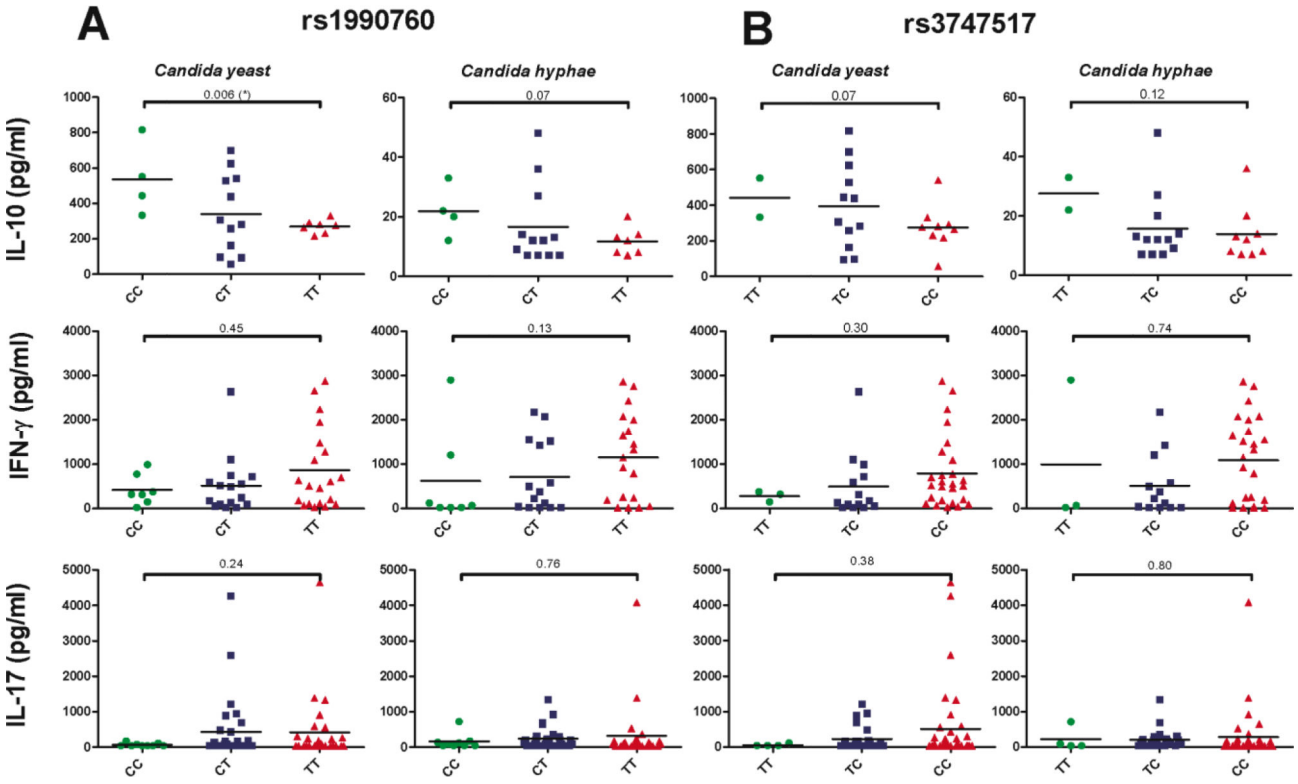


Figure 4. ***IFIH1*** **missense SNP genotypes correlate with** ***Candida*****-induced cytokine levels**

PBMCs from healthy volunteers with different genotypes for (**A**) rs1990760 (candidemia risk allele T) and (**B**) rs3747517 (candidemia risk allele C) were stimulated *in vitro* with either *C. albicans* yeast or hyphae. Cytokine levels (scatterplots with mean indicated) were measured after 24 hours (IL-10) or 7 days (IL-17 and IFN-γ) by enzyme linked immunosorbent assay (ELISA). *P* values were calculated using the Mann-Whitney *U* test comparing cytokine levels of the two homozygous genotypes.

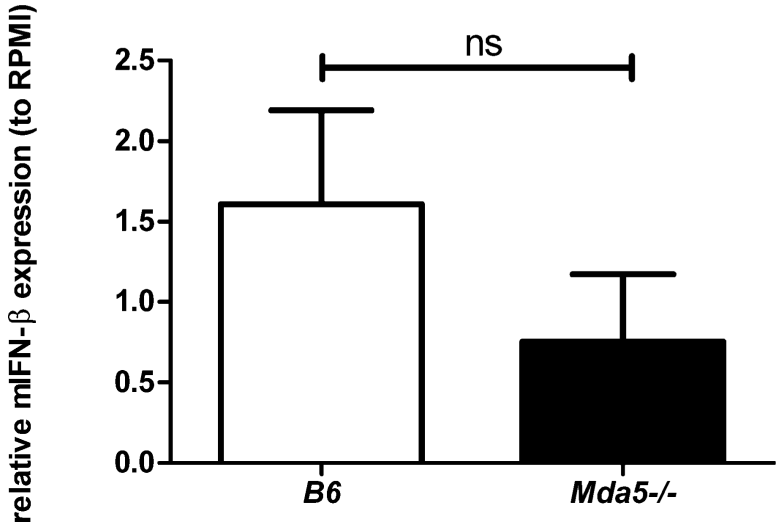


Figure 5.

Relative gene expression (mean ± SEM) of mouse interferon β (mIFN-β) in splenocytes isolated from control B6 control mice (C57BL/6J) and Mda5 knockout mice, upon stimulation with *C. albicans* hyphae (10^6/ml) (n=5/group). The *P* value was not significant at $\alpha < 0.05$ (calculated using the Welch-corrected t test).

Table 1

KEGG pathway enrichment for genes that are specifically induced in macrophages stimulated with wild-type *Candida* for 24 hours.

KEGG identifier	Pathway	*P* value	Fold enrichment	Genes
hsa04622	*RIG-I-like receptor signaling pathway*	4.3×10^{-3}	*11.5*	*IFIH1, ISG15, IL8, TRIM25*
hsa04060	Cytokine-cytokine receptor interaction	6.6×10^{-3}	4.7	OSM, TNFSF13B, IL8, CXCR4, CCL3L1, CCL5
hsa04612	Antigen processing and presentation	6.6×10^{-3}	9.8	TAP2, TAP1, HSPA6, HSPA1B
hsa04062	Chemokine signaling pathway	1.1×10^{-2}	5.4	IL8, CXCR4, CCL3L1, CCL5, STAT1
hsa04621	NOD-like receptor signaling pathway	3.4×10^{-2}	9.8	IL8, RIPK2, CCL5
hsa05120	Epithelial cell signaling in Helicobacter pylori infection	4.0×10^{-2}	9.0	IL8, HBEGF, CCL5

Enrichment for KEGG pathway components [49] was determined using the functional annotation tool of the DAVID suite [50]. Background: all human genes.

Table 2

Selection of SNPs in the *FAP-IFIH1-GCA-KCNH7* LD region that are significantly associated with susceptibility to candidemia

SNP	Immunochip		Closest gene(s)	Alleles (dbSNP)	Functional class (AA change)	BH-corrected genotypic *P* value
rs984971	imm_2_162932767		*GCA* | *KCNH7*	A/G	intergenic	6.9×10^{-4}
	Genotypes	*GG*	*GA*	*AA*		
	Controls	25 (14.2%)	103 (58.5%)	48 (27.3%)	$P = 2.2 \times 10^{-5}$	
	Cases	25 (11.0%)	89 (39.2%)	113 (49.8%)		
	Alleles	*G*	*A**			
	Controls	153 (43.5%)	199 (56.5%)	$P = 2.2 \times 10^{-4}$		
	Cases	139 (30.6%)	315 (69.4%)	OR, G vs. A = 0.57 (0.43 – 0.77)		
rs1990760	imm_2_162832297		*IFIH1*	C/T	missense (Ala946Thr)	3.0×10^{-3}
	Genotypes	*CC*	*CT*	*TT*		
	Controls	31 (17.6%)	99 (56.3%)	46 (26.1%)	$P = 1.9 \times 10^{-4}$	
	Cases	37 (16.3%)	87 (38.3%)	103 (45.4%)		
	Alleles	*C*	*T**			
	Controls	161 (45.7%)	191 (54.3%)	$P = 3.7 \times 10^{-3}$		
	Cases	161 (35.5%)	293 (64.5%)	OR, C vs. T = 0.65 (0.49 – 0.87)		
rs3747517	imm_2_162837070		*IFIH1*	T/C (A/G)	missense (His843Arg)	8.7×10^{-3}
	Genotypes	*TT*	*TC*	*CC*		
	Controls	12 (6.8%)	88 (50.0%)	76 (43.2%)	$P = 1.4 \times 10^{-3}$	
	Cases	20 (8.8%)	73 (32.2%)	134 (59.0%)		
	Alleles	*T*	*C**			
	Controls	112 (31.8%)	240 (68.2%)	$P = 3.3 \times 10^{-2}$		
	Cases	113 (24.9%)	341 (75.1%)	OR, T vs. C = 0.71 (0.52 – 0.97)		

Genotypic and allelic associations were assessed using the Fisher's exact test. *P* values are shown next to the corresponding contingency tables. Odds ratios (OR, with 95% confidence intervals) are reported for the allelic association tests and represent the odds of disease for individuals carrying the non-risk allele versus the risk allele. Risk alleles are denoted by an asterisk. BH-corrected genotypic *P* value: Benjamini-Hochberg correction of the genotypic association test *P* values for testing multiple SNPs (64 in total). Immunochip: the identifier of the SNP on the Immunochip SNP array. Alleles: the alleles measured with the Immunochip, and the complementary alleles reported by dbSNP (build 138), if they are different. The table shows the top SNP in the LD region, along with the only two significant missense SNPs. All SNPs tested are in Hardy–Weinberg equilibrium in the controls ($P > 1 \times 10^{-3}$). See **Table S1** for the full list of significant SNPs.