Immunogenetic profiling to predict risk of invasive fungal diseases: where are we now?

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Immunogenetic profiling to predict risk of invasive fungal diseases: where are we now?

Running head: Immunogenetics of invasive fungal diseases

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Abstract

Invasive fungal diseases remain nowadays life-threatening conditions affecting multiple clinical settings. The onset of these diseases is dependent on numerous factors, of which the "immunocompromised" phenotype of the patients is the more often acknowledged. However, and despite comparable immune dysfunction, not all patients are ultimately susceptible to disease, suggesting that additional risk factors, likely of genetic nature, may also be important. In the last years, genetic variants in several immune-related genes have also been proposed as major determinants of the susceptibility pattern of high-risk patients to invasive fungal diseases. Altogether, these findings highlighted the crucial significance of the individual genetic make-up in defining susceptibility to infection, providing a compelling rationale for the introduction of the immunogenetic profile as a risk prediction measure that may ultimately help to guide clinicians in the use of prophylaxis and preemptive fungal therapy in high-risk patients.

Introduction

Fungal diseases are epidemiologically characteristic of high-risk groups. These include patients with hematological malignancies, prolonged and severe neutropenia, undergoing corticosteroid therapy or submitted to solid organ or stem cell transplantation, among others. However, besides the relative risk conferred by the "immunocompromised" phenotype as a result of the underlying condition or treatment scheme, certain individuals with specific, genetically-determined, immune defects are also more vulnerable to infection.

Over the last decades, considerable advances have been made in understanding the molecular bases determining disparate susceptibilities to infectious diseases in humans. Monogenetic defects underlying primary immunodeficiencies (PIDs) have been classically associated with predisposition to a restricted spectrum of fungal pathogens causing clinically defined manifestations. For example, the hyper-IgE syndrome (HIES) or the autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome are usually correlated with susceptibility to superficial fungal infections, most remarkably chronic mucocutaneous candidiasis (CMC). On the other hand, chronic granulomatous disease (CGD), an inherited disorder of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that impairs the production of reactive oxygen species, predisposes to several recurrent life-threatening infections, of which invasive aspergillosis (IA) is the most distinctive fungal disease.

In addition to PIDs, other factors modifying risk of fungal disease are often genetic polymorphisms. These have a substantial allele prevalence in the general population but, under select contexts of immunocompromise typical of many clinical settings, may translate into a further severity of the immunological dysfunction, ultimately rendering these individuals more prone to disease. In this article, we review the contribution of genetic polymorphisms of the immune system to susceptibility to invasive fungal diseases and discuss how this knowledge may be used for the design of individually tailored approaches to be integrated in clinical practice.

Genetic defects of PRRs and susceptibility to invasive fungal diseases

Invasive fungal diseases are usually devastating conditions associated with high mortality rates and that affect a broad range of patients with apparently disparate underlying risk factors (e.g. allogeneic stem cell transplant recipients). Given the need to predict risk for disease in these patients and design effective preventive strategies, a relatively large number of studies have investigated the association between genetic variants of pattern recognition receptors (PRRs) or inflammatory mediators and risk of invasive fungal diseases (Carvalho *et al.*, 2010b) (Figure 1).

Toll-like receptors (TLRs)

TLRs participate in the recognition of microbial structures and in the initiation of inflammatory and antimicrobial host defenses. They are expressed in several cell types including, but not exclusively, monocytes, macrophages, dendritic cells and neutrophils, and can be predominantly expressed on the cell surface (TLR1, -2, -4, -5 and -6) or retained intracellularly in endosomes (TLR3, -7, -8 and -9). Recent evidence also attested a major contribution of TLRs present at epithelial surfaces to antimicrobial defense and immunosurveillance (Weindl *et al.*, 2007; de Luca *et al.*, 2010). Distinct TLR ligands are able to mediate disparate effector responses through the same receptor, a phenomenon in part explained by the selective usage of adapter molecules such as myeloid differentiation factor 88 (MyD88) and Toll/IL-1 receptor (TIR) domain-containing adapter inducing interferon-β (TRIF). Receptor engagement eventually culminates in the activation of transcription factors such as nuclear factor κB (NF-κB) and members of the interferon regulatory factor (IRF) family, which successively induce gene expression and production of various cytokines, chemokines and molecules required for antigen presentation or costimulation.

The early finding that Toll-deficient *Drosophila* were unable to mount effective antifungal responses and were highly susceptible to infection by *Aspergillus fumigatus* highlighted a similar participation of mammalian TLRs in antifungal immunity. In fact, TLR2, -4 and -9 function has

been categorically demonstrated to contribute to host responses against fungi both in mice and in humans (Cunha *et al.*, 2010c). Given its crucial role in the immune response to fungi, TLR4 has been regarded as one major potential repository of genetic variability contributing to susceptibility to fungal diseases. For this reason, most studies performed to date have focused on the highly polymorphic *TLR4* gene, in which two nonsynonymous polymorphisms – D299G and T399I – have been linked with blunted responses to inhaled lipopolysaccharide (Arbour *et al.*, 2000).

A donor haplotype in *TLR4* consisting of the above-mentioned polymorphisms was disclosed as an independent predictive factor for IA among unrelated hematopoietic stem cell transplant (HSCT) recipients (Bochud *et al.*, 2008). Although alternative explanations for this association have been proposed, namely the possible interaction of TLR4 with cytomegalovirus (CMV) (Cervera *et al.*, 2009) or antifungal drugs (Levitz *et al.*, 2009), the D299G and T399I polymorphisms have been linked with susceptibility to pulmonary aspergillosis in non-transplanted patients, who are not susceptible to CMV disease and usually do not receive antifungal prophylaxis (Carvalho *et al.*, 2008). In addition, we have reported an association of the *TLR4* haplotype with fungal colonization following T-cell-depleted transplantation, although without increased risk for invasive disease (Carvalho *et al.*, 2009). A previous study had also failed to associate *TLR4* polymorphisms with IA in HSCT recipients, despite evidence pointing to a role of genetic variants in *TLR1* and *TLR6* (Kesh *et al.*, 2005). These discrepancies between studies further stress that the contribution of genetic variants to IA may depend on several factors, such as the type of transplant and associated demographic and clinical co-variables. Uncovering the biological implications of *TLR4* polymorphisms in the immune response to *Aspergillus* would ultimately strengthen the clinical repercussions of these findings. It is worth mentioning that polymorphisms in *TLR2* and *TLR9* were not associated with IA (Bochud *et al.*, 2008; Carvalho *et al.*, 2009), although a common promoter polymorphism in *TLR9* (-1237T>C) was shown to predispose to allergic bronchopulmonary aspergillosis (Carvalho *et al.*, 2008).

However, it also holds true that the absence of association should always be interpreted in the scope of the limited power of the existing studies.

The *TLR4* haplotype had also been suggested to contribute to a higher risk of *Candida* bloodstream infection (Van der Graaf *et al.*, 2006). However, this underpowered report has been recently challenged by a study from the same group in a large cohort of patients with invasive candidiasis (IC), which excluded any role of these genetic variants in susceptibility to this disease (Plantinga *et al.*, 2009a). Instead, polymorphisms in *TLR1* were shown to be strong predictive markers for IC and functional assays demonstrated that the increased risk was associated with impaired production of cytokines such as IL-1β, IL-6 and IL-8. Although this provides proof of concept that TLR1 plays a major role in the pathogenesis of IC, it is of note that this study dealt only with nonsynonymous polymorphisms and thus, does not exclude additional genetic variability potentially impacting susceptibility to this disease.

In addition, and despite no studies have revealed an association between genetic variants in *TLR2* and candidiasis, the nonsynonymous polymorphism R753Q was shown to modulate cytokine release during *Candida* sepsis in intensive care unit patients (Woehrle *et al.*, 2008). The extent to which the deregulated cytokine production contributes to susceptibility to candidiasis is however still not clear.

C-type lectin receptors (CLRs)

Even though the signaling pathways elicited by TLRs are crucial for the control of fungal infection (Bellocchio *et al.*, 2004), a pivotal role for dectin-1 as the prototype of innate non-TLR signaling pathway for antifungal sensing has been highlighted (Brown, 2006). Dectin-1 is a CLR that specifically recognizes the cell wall carbohydrate β-(1,3)-glucan of many fungi and mediates cell activation, cytokine production and a variety of antifungal responses either through the spleen tyrosine kinase Syk/cytoplasmic caspase recruiting domain 9 (CARD9) (LeibundGut-Landmann *et al.*, 2007) or Raf-1 (Gringhuis *et al.*, 2009) pathways.

Polymorphisms in the gene encoding for dectin-1 have also been recently addressed as potential predictive factors for the incidence of fungal diseases. In particular, the early stop codon polymorphism Y238X has been found to associate with recurrent mucocutaneous fungal infections (Ferwerda *et al.*, 2009) and to contribute to *Candida* colonization after HSCT (Plantinga *et al.*, 2009b). This susceptibility phenotype was correlated with the generation of a truncated dectin-1 protein unable to target the membrane, thereby restraining β-glucan binding by monocytes and resulting in defective cytokine production upon receptor engagement, in particular IL-17 (Ferwerda *et al.*, 2009; Plantinga *et al.*, 2009b). Interestingly, a similar susceptibility pattern to mucocutaneous infections related to a deficit in IL-17 production was also found in a family carrying loss-of-function mutations in *CARD9* (Glocker *et al.*, 2009), suggesting that disruption of dectin-1 signaling and concomitant failure to produce IL-17 in response to *Candida* critically impairs mucosal antifungal defense. Accordingly, the clinical manifestation of CMC was also recently found to rely on genetic defects in *IL17F* and *IL17RA* (Puel *et al.*, 2011).

Work from our group has also demonstrated that dectin-1 deficiency contributed as well to an increased susceptibility to IA following HSCT, an association which relied on either recipient or donor genetic make-ups and to be synergistically increased in polymorphic recipient/donor pairs (Cunha *et al.*, 2010a). Another study has instead proposed a limited influence of the Y238X polymorphism on susceptibility to IA, in particular in non-HSCT patients (Chai *et al.*, 2011). This study however failed to address the likely confounding effects of population stratification on their conclusions given the enrollment of admixed heterogeneous patient populations. Larger, well-designed studies, preferably performed on consecutive patients, are ultimately required to clarify the role of the Y238X polymorphism in both HSCT and non-HSCT patients at risk of IA.

Although dectin-1 has been regarded as one major innate receptor leading to T helper 17 (Th17) activation in response to *Aspergillus* (Werner *et al.*, 2009), production of IFN-γ and IL-10 by dectin-1-deficient peripheral blood mononuclear cells, more than IL-17, was found to be

impaired (Cunha *et al.*, 2010a). Interestingly, polymorphic macrophages displayed instead adequate responses to the fungus (Chai *et al.*, 2011). This suggests that pulmonary macrophages, in view of their role as primary line of defense, retain their ability to properly respond to *Aspergillus* ligands even in the absence of functional dectin-1, likely due to the inherent redundancy of the several PRRs (e.g. mannose receptor, TLR2, TLR4, etc.). Moreover, the contribution of recipient dectin-1 deficiency to the high risk of infection also reflects the crucial role of nonhematopoietic dectin-1 in the induction of immune protection to the fungus, highlighting the overlapping mechanisms of immune resistance and tolerance that are conditioned by the hematopoietic/nonhematopoietic compartmentalization.

Other PRRs

The collectin subfamily of lectins includes members such as mannose-binding lectin (MBL), pentraxin 3 (PTX3) and the lung surfactant proteins (SPs). Collectins are able to bind to fungi and activate the complement system leading to their opsonization or direct killing. Among this class of PRRs, MBL is the most extensively dissected member from a genetic point of view. A combination of structural and promoter polymorphisms is known to exist in the *MBL2* gene and each may define the levels of functional protein (Garred, 2008). For this reason, since it was first reported, MBL deficiency has been consistently associated with increased susceptibility to infections, particularly when adaptive immunity is impaired. Accordingly, reduced levels of MBL have been recently correlated with the incidence of acute IA in non-HSCT immunocompromised patients, despite no causal nature was attributed to MBL polymorphisms (Lambourne *et al.*, 2009). Nevertheless, donor *MBL2*^{low} genotypes (O/O or LXA/O) have been identified as important predictive factors of increased incidence of invasive fungal diseases after HSCT (Granell *et al.*, 2006). Similarly, the nonsynonymous polymorphism D105G in recipient MBL-associated serine protease 2 gene (*MASP2*), known to hinder MBL function, was also linked with invasive fungal disease after HSCT (Granell *et al.*, 2006). However, the later finding deserves additional validation owing to the low number of recipients with the *MASP2* variant.

In addition to MBL, genetic mapping analysis of the survival data of an immunocompromised mice model has also allowed the identification of plasminogen, a regulatory molecule that binds to *Aspergillus*, as a suitable candidate gene for aspergillosis susceptibility (Zaas *et al.*, 2008). The clinical translation of these findings acknowledged the nonsynonymous polymorphism D472N in the human gene encoding for plasminogen to influence the risk of developing IA in HSCT recipients, particularly late after transplantation. Besides shedding light into the role of the fibrinolytic system in the pathogenesis of IA, this computational approach identified a novel and biologically plausible candidate gene for susceptibility to IA, therefore validating its future use in the identification of less obvious fungal disease-related genes.

Cytokines/chemokines

Cytokines mediate the inflammatory and adaptive immune responses to pathogens. Whereas inflammation may serve to limit infection, when heightened, it may significantly contribute to pathogenicity, as documented by the elevated incidence of severe fungal infections in patients with immune reconstitution syndrome (Singh and Perfect, 2007). It is not surprising therefore that patients with inborn errors in the IL-12/IFN-γ axis, unable to mount proper inflammatory responses, are highly susceptible to disseminated fungal diseases such as paracoccidioidomycosis (PCM) (Moraes-Vasconcelos *et al.*, 2005) and histoplasmosis (Zerbe and Holland, 2005).

Genetic variants in cytokine genes have been considered crucial factors determining vulnerability to fungal infections, particularly aspergillosis. In this regard, IL-10 has been considered one major candidate for genetic studies as its production has been suggested to be largely genetically-determined, in part due to the promoter polymorphism -1082G>A. Indeed, patient *IL10^{low}* genotypes were shown to confer protection from IA in both HSCT (Seo *et al.*, 2005) and non-HSCT (Sainz *et al.*, 2007a) patients. Despite the fact that no actual correlation with serum levels was established in these studies, the validity of these associations is fairly

reinforced by the finding that *IL10*^{high} genotypes were instead linked with increased colonization with *Aspergillus* and allergic disease in patients with cystic fibrosis (Brouard *et al.*, 2005). Similarly, increased production of IL-10 resulting from *IL10*^{high} genotypes has also been shown to contribute to the development of chronic fungal infections such as PCM (Bozzi *et al.*, 2006). Hence, and regardless of the lack of functional data definitely correlating *IL10* polymorphisms with impaired immune responses to *Aspergillus*, the consistency of the associations based on genotype-defined IL-10 levels reflects the likely role of these polymorphisms in determining resistance versus susceptibility to aspergillosis.

A small number of other studies have addressed the role of polymorphisms in additional cytokines and/or their receptors as predisposing factors to IA. For example, and despite analysis of individual locus failed to associate variants in the genes encoding for IL-1α, IL-1β and IL-1R antagonist (IL-1Ra) with IA, a polymorphism haplotype in this gene cluster (VNTR2/-889C/-511T) was nevertheless strongly associated with susceptibility to this disease in hematological patients (Sainz *et al.*, 2008). Work from the same group has also demonstrated a crucial contribution of polymorphisms in the TNF receptor 1 (*TNFR1*) and 2 (*TNFR2*) to an increased susceptibility to IA in non-HSCT patients (Sainz *et al.*, 2007b; Sainz *et al.*, 2010). In this case, and particularly regarding TNFR1, the observed susceptibility to IA was correlated with defective receptor expression, suggesting a crucial contribution of TNF-α signaling in the immune response to *Aspergillus*, at least in this particular setting of patients.

In addition, we have found that the functional polymorphism R381Q affecting the receptor for IL-23 (IL-23R) instead conferred a protective effect regarding IA and correlated with improved survival of HSCT recipients (Carvalho *et al.*, 2010a). Functionally, this specific polymorphism has been demonstrated to promote a deficient activation of IL-23-driven Th17 responses (Cunha *et al.*, 2010b). Interestingly, whereas committed effector Th17 cells derived from subjects harboring R381Q stimulated with IL-23 had an impaired production of IL-17, highly purified naïve T-cells did not, suggesting that the consequences of the polymorphism may be exclusively reflected in the function of differentiated Th17 cells.

Recently, data has also pointed to the relevance of genetic variants in chemokine genes in host susceptibility to fungal diseases. A large-scale screening of polymorphisms led to the finding of a haplotype in CXC chemokine ligand 10 (*CXCL10*) resulting in increased susceptibility to IA in HSCT recipients (Mezger *et al.*, 2008). Mechanistically, immature wild-type dendritic cells exposed to *A. fumigatus* showed a markedly increase in *CXCL10* expression, in contrast to those harboring the risk haplotype. In this regard, it is also interesting to note that patients who survived IA had significantly higher CXCL10 levels in comparison to healthy controls.

Altogether, and despite the need for replication studies in independent cohorts of patients, these findings point to the importance of maintaining a finely orchestrated balance between pro- and anti-inflammatory signals, fundamental for the immune system to effectively attack and eliminate pathogenic fungi, suggesting that the disruption of this equilibrium may ultimately underlie an increased predisposition to IA.

Immunogenetics of invasive fungal diseases: what does the future hold?

The dissection of the genetic bases underlying susceptibility to infectious diseases inspires a very active, yet complex, field of research. As the awareness of the individual genetic make-up may hold the key to expose unsuspected risk factors for these diseases, the current understanding of the immunological network involved in the immune response to pathogens needs to be addressed also from a genetic point of view. Given that the modulation of host immune responses has been regarded as a potential therapeutic target for the eradication of fungal diseases, knowing each individual's immunogenetic profile, together with the application of promising therapeutic strategies such as siRNA delivery (Bonifazi *et al.*, 2010), could prove critical for the design of novel, effective fungal vaccines capable of targeting and exerting control over the outcome of specific signalling pathways.

Although many of the studies published so far still lack replication, a step forward in this field would be provided by large-scale translational and clinical studies validating the data obtained from the genetic analyses. This would support the use of genetic screening of at-risk

patients and would ultimately allow the individualization of prophylaxis and treatments by means of targeted and patient-tailored approaches likely improving the management and outcome of these severe, often fatal diseases.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Figure legends

Figure 1. Genetic polymorphisms of pattern recognition receptors (PRRs) and inflammatory mediators associated with susceptibility to invasive fungal diseases.

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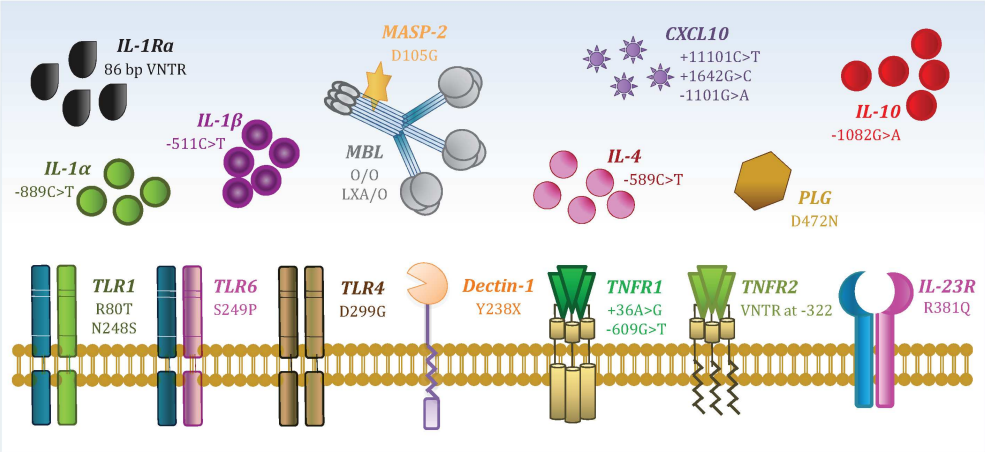

Figure 1. Genetic polymorphisms of pattern recognition receptors (PRRs) and inflammatory mediators associated with susceptibility to invasive fungal diseases.
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