Universidade do Minho
Escola de Ciências da Saúde

Agostinho Albérico Rodrigues de Carvalho

Management of fungal infections: diagnosis and human genetic susceptibility

Infecções fúngicas: diagnóstico e susceptibilidade genética humana

Maio de 2008

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Tese de Doutoramento
Ciências da Saúde - Ciências Biológicas e Biomédicas

Trabalho efectuado sob a orientação do
Doutor Fernando José dos Santos Rodrigues

Maio de 2008

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THE WORK PRESENTED IN THIS DISSERTATION WAS MAINLY DONE WITHIN THE MICROBIOLOGY AND INFECTION RESEARCH DOMAIN IN THE LIFE AND HEALTH SCIENCES RESEARCH INSTITUTE (ICVS), SCHOOL OF HEALTH SCIENCES, UNIVERSITY OF MINHO. PART OF THE WORK WAS ALSO DONE IN THE DEPARTMENT OF EXPERIMENTAL MEDICINE, UNIVERSITY OF PERUGIA. THE FINANCIAL SUPPORT WAS GIVEN BY FUNDAÇÃO PARA A CIÊNCIA E TECNOLOGIA BY MEANS OF A GRANT, SFRH/BD/11837/2003.

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ABSTRACT

Invasive fungal infections represent nowadays a major public health problem with associated high mortality rates. The lack of adequate diagnostic methods, together with the fact that many emerging fungal species are resistant to the currently available antifungal agents, contributes to the profound impact of these diseases in the health care systems, especially when dealing with immunocompromised patients. Taking this into consideration, the development of novel diagnostic applications has been considered a critical issue in recent years. We described a multiplex PCR-based strategy allowing the identification of eight of the most clinically relevant *Candida* species. The strategy, based on the amplification of fragments from the internal transcribed spacer regions of the ribosomal RNA genes, was shown to present both high specificity and sensitivity, in addition to other attractive features, including the individual discrimination of species present in mixture and the direct identification from clinical specimens, characteristics reinforcing the potential clinical application of the method.

In addition to the need of more satisfactory diagnostic methods, the understanding of the host-fungi interaction, namely at the level of host genetic susceptibility, is critical to advance the knowledge regarding these infections and, in particular, the individual risk factors predisposing to them. Differences in human susceptibility to infectious diseases have been widely described with recent examples focusing on genetic variations within genes of the innate immune system, such as Toll-like receptors (TLRs), which may alter host-pathogen defence mechanisms, thus affecting susceptibility to infectious diseases, and in particular, fungal infections. Taking this into account, we developed a simple and rapid method based in the bi-directional PCR amplification of specific alleles (Bi-PASA) for genotyping known sequence variants in the TLR genes to be used in the forthcoming association studies regarding genetic susceptibility to fungal infections. The development of this methodology also allowed us to perform a characterization of the general Portuguese population regarding these polymorphisms, that can be used in future association studies, besides providing valuable information regarding stratification of patients most at risk of infection.

Following the demonstration of the usefulness of Bi-PASA, we investigated the potential association between polymorphisms in the TLR genes and susceptibility to non-invasive forms of pulmonary aspergillosis. A significant association was observed between the presence of Asp299Gly (*TLR4*) and chronic cavitary pulmonary aspergillosis. In the same way, this variation

was also linked with fungal colonization in the haematopoietic stem cell transplantation (HSCT) setting, suggesting that an abnormal TLR4 extracellular domain may be impairing the recognition of the fungus, thus contributing to an increased predisposition to these diseases. However, the same polymorphism was previously shown to have a protective role against invasive aspergillosis in HSCT patients. Thus, as shown for hyper-inflammatory states, such as atherosclerosis, impairments in the production of inflammatory cytokines contributing to disease susceptibility may be compensating the effect of the defective TLR4. Furthermore, susceptibility to another form of pulmonary aspergillosis, allergic bronchopulmonary aspergillosis, as well as viral infection in the HSCT setting, were shown to be associated with T-1237C (*TLR9*), highlighting the divergent function of TLRs in the pathogenesis of these infections.

A shared susceptibility mechanism involving the T-1237C polymorphism in the promoter region of *TLR9* was also observed to predispose to the development of non-Hodgkin lymphoma (NHL). This disease includes a set of heterogeneous lymphoproliferative malignancies often associated with an altered immunological function of the host and chronic inflammatory type of infections, in which TLR9 was already shown to play a critical role. The T-1237C polymorphism introduces a regulatory site that is *trans*-activated by the IL-6-dependent transcription factor IL-6 response element binding protein (IL-6 RE-BP), thus resulting in increased expression of *TLR9*. TLR9 activation of B lymphocytes leads to yet increased gene expression levels and sequentially to augmented proliferation rates, as well as higher production of IL-6. This was shown to result in a TLR9 activation loop leading to B lymphocyte-specific uncontrolled proliferation, making these cells more prone to acquire transforming mutations associated with the development of NHL. Besides uncovering a major risk factor for the development of NHL, the presented information has important implications on the recent usage of CpG agonists on several therapeutic strategies in cancer and autoimmune diseases.

In summary, we have contributed to show that management of fungal infections, both invasive and non-invasive, involves not only the development of more satisfactory diagnostic procedures, but also considerable attention that has to be given to individual genetic variants that, as we showed, are able to alter susceptibility to these infections. These findings have potential relevance for the stratification of patients most at risk, not only of fungal infections, but also to diseases of other aetiological natures, whose pathogenesis share common signalling/activation pathways such as those presented by the TLRs.

RESUMO

Actualmente, as infecções fúngicas invasivas representam um importante problema de saúde pública, sendo responsáveis por elevadas taxas de mortalidade. A falta de métodos de diagnóstico adequados, associada à resistência que muitas das espécies fúngicas emergentes apresentam aos antifúngicos disponíveis, contribui para um profundo impacto destas doenças, especialmente no caso dos doentes imunocomprometidos. Tendo em consideração estes aspectos, o desenvolvimento de novas metodologias de diagnóstico tem sido encarado como uma necessidade prioritária. Neste sentido, desenvolveu-se uma nova estratégia, baseada em PCR multiplex, que permite a identificação de oito das espécies de *Candida* mais relevantes em termos clínicos. Este método, baseado na amplificação de fragmentos dos genes de RNA ribossomal, apresenta uma elevada especificidade e sensibilidade. A sua potencial aplicação clínica é ainda reforçada por outras características, nomeadamente a discriminação individual de espécies presentes em co-infecção e a identificação directa a partir de espécimes clínicos.

Juntamente com a necessidade de desenvolvimento de métodos de diagnóstico mais satisfatórios, a compreensão da interacção hospedeiro-fungo, nomeadamente ao nível da susceptibilidade genética humana, é essencial para o avanço do conhecimento destas infecções. Diferenças na susceptibilidade humana a doenças infecciosas têm sido descritas, associadas nomeadamente a variações em genes do sistema imunológico inato, como os receptores "Toll-like" (TLRs). Recentemente, verificou-se que estas variações podem alterar os mecanismos de defesa antimicrobianos, afectando a susceptibilidade a doenças infecciosas. Assim, tendo em conta estes aspectos, desenvolvemos um método simples e rápido para genotipar polimorfismos nos genes TLR, de forma a aplicá-lo na análise da susceptibilidade a infecções fúngicas nos estudos de associação subsequentes. O desenvolvimento desta metodologia permitiu ainda a caracterização da população Portuguesa em relação a estes polimorfismos, dados que poderão ser usados em estudos futuros, para além de fornecer informação pertinente acerca da estratificação de pacientes com maior risco de desenvolverem infecções.

Após a demonstração da utilidade da técnica de Bi-PASA, investigámos a potencial associação entre polimorfismos nos genes TLR e a susceptibilidade a formas não-invasivas de aspergilose pulmonar. De acordo com os resultsdos obtidos, observou-se uma associação entre a presença de Asp299Gly (*TLR4*) e a aspergilose pulmonar crónica cavitária. De forma idêntica, verificou-se uma associação entre o mesmo polimorfismo e a colonização fúngica em doentes

sujeitos a transplante de células estaminais hematopoiéticas (HSCT), sugerindo que um domínio extracelular anómalo de TLR4 pode limitar o reconhecimento do fungo, contribuindo para uma maior predisposição para estas doenças. Contudo, a mesma variação apresentou um papel protector em relação a aspergilose invasiva, sugerindo que uma redução na produção de citocinas pró-inflamatórias poderá equilibrar o defeito anterior, tal como foi anteriormente demonstrado para estados patológicos de hiper-inflamação, como a aterosclerose. Adicionalmente, demonstrámos que o polimorfismo T-1237C (*TLR9*) estava associado a uma maior susceptibilidade a aspergilose broncopulmonar alérgica, uma outra forma de aspergilose pulmonar, assim como a infecções virais em pacientes HSCT, realçando a função divergente dos TLRs na patogénese destas doenças.

Um mecanismo de susceptibilidade partilhado envolvendo o polimorfismo T-1237C surgiu também como predispondo para o desenvolvimento de linfoma não-Hodgkin (NHL). Este inclui um conjunto de doenças linfoproliferativas, frequentemente associadas a alterações imunológicas do hospedeiro e infecções associadas a respostas inflamatórias crónicas, nas quais já foi descrito um papel importante de TLR9. O polimorfismo T-1237C introduz um local de regulação que é *trans*-activado por um factor de transcrição dependente de IL-6, resultando numa elevada expressão de *TLR9*. A activação deste receptor em linfócitos B leva a um aumento da sua expressão genética e, consequentemente, a taxas de proliferação mais elevadas, assim como à produção excessiva de IL-6. Estas alterações, juntamente com uma activação persistente de TLR9, culminam numa proliferação descontrolada dos linfócitos B, tornando estas células mais susceptíveis à aquisição de mutações transformantes associadas com o desenvolvimento de NHL. Para além de termos evidenciado o polimorfismo T-1237C como um factor de risco para o desenvolvimento de NHL, a informação resultante apresenta ainda importantes implicações no uso, recentemente preconizado, de agonistas de TLR9 em diversas estratégias terapêuticas, nomeadamente cancro e doenças autoimunes.

Em resumo, demonstrámos que a abordagem das infecções fúngicas deve abranger não só um interesse particular no fungo, através do desenvolvimento de métodos de diagnóstico mais eficazes, mas também uma atenção considerável às variações genéticas individuais que, como demonstrámos, podem modular a susceptibilidade a estas infecções. Os resultados aqui apresentados têm uma potencial relevância na estratificação de pacientes com maior risco para infecções fúngicas, bem como doenças de outras etiologias, cuja patogénese partilha vias comuns de sinalização/activação, como as apresentadas pelos TLRs.

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OBJECTIVES AND OUTLINE OF THE THESIS

The work presented throughout this dissertation was developed in the context of the projects *"Recognition mechanisms of pathogen-associated molecular patterns: implications in susceptibility to infections relevant to public health"* (funded by Fundação Calouste Gulbenkian, Programa de Apoio à Prevenção e Rastreio de Doenças Infecciosas e Oncológicas Socialmente Relevantes) and *"A multidisciplinary study of invasive fungal infections in immunosuppressed patients: host susceptibility to fungi versus fungi resistance to drugs"* (funded by Fundação para a Ciência e Tecnologia).

The fact that most of the currently available diagnostic procedures for invasive fungal infections are still not satisfactory contribute to the high mortality rates associated to these infections. Additionally, the lack of knowledge concerning the host-pathogen interaction, namely at the level of host genetic susceptibility, led us to propose the following objectives for this thesis: (1) to develop molecular methodologies with potential clinical and epidemiological application in the identification of relevant fungal pathogens, providing an alternative to currently available procedures (2) to study the potential associations between polymorphisms in TLR genes and susceptibility to fungal infection, both invasive and non-invasive forms, and (3) to elucidate the functional impact of the abovementioned polymorphisms in the mechanisms of disease susceptibility.

The present dissertation is organized in 8 different chapters, with the last chapter consisting of the reference list. Chapter 1 consists of a general introduction, presenting a review of the current knowledge on the host-pathogen interaction, with particular emphasis on the innate immune response and its genetic variants, and the manner(s) in which they may predispose to infectious disease, more specifically fungal infection.

In chapter 2, the work "Multiplex PCR identification of eight clinically relevant *Candida* species" is presented, focusing on the development of a rapid, simple and effective PCR-based methodology to specifically detect and identify clinically relevant *Candida* species. The developed method presents several advantages over currently available diagnosis procedures, including high specificity and sensitivity, as well as reduced time required for identification. Altogether, these characteristics point to a highly advantageous method with potential application in both clinical diagnosis and epidemiological studies.

In chapter 3, the work "Study of disease-relevant polymorphisms in the *TLR4* and *TLR9* genes: a novel method applied to the analysis of the Portuguese population" is presented, showing the development of a genotyping method based on allele-specific PCR and its application in the characterization of the general Portuguese population regarding polymorphisms in TLRs, thus providing valuable information that can be potentially used to stratify risk patients with increased susceptibility to infection.

In chapter 4, the work "Polymorphisms in Toll-like receptor genes and susceptibility to pulmonary aspergillosis" is presented, showing a case-control association study between polymorphisms in TLRs and susceptibility to non-invasive forms of pulmonary aspergillosis. The findings presented in this chapter reinforce the importance of innate immunity, and in particular genetic variations in TLR genes, in the pathogenesis of different forms of pulmonary aspergillosis.

In chapter 5, the work "Polymorphisms in Toll-like receptor genes and susceptibility to infections in allogeneic stem cell transplantation" is presented, describing the results of a case-control association study regarding the contribution made by polymorphisms in TLRs to susceptibility to fungal and/or viral infection in patients undergoing haematopoietic stem cell transplantation (HSCT). The results presented in this chapter highlight the divergent function of TLRs in the pathogenesis of opportunistic infections, in particular in the HSCT setting.

In chapter 6, the work "Risk of developing non-Hodgkin lymphoma associates with the T-1237C polymorphism in the *TLR9* promoter" is presented, illustrating the role of a *TLR9* polymorphism in the development of non-Hodgkin lymphoma (NHL). This line of research was initiated during the studies depicted in chapter 5, upon the observation that this polymorphism was highly prevalent in NHL patients. In particular, data is presented regarding the functional impact of this polymorphism and the molecular mechanisms underlying increased susceptibility to NHL, suggesting an important role for TLR9 in the development of this malignancy.

In chapter 7, concluding remarks are presented bringing together Chapter 2, 3, 4, 5 and 6 in the context of the initially proposed objectives. In particular, the importance of TLR polymorphisms in susceptibility/resistance to fungal infections, as well as the significance of the shared pathways affected by these polymorphisms and their role in the predisposition to diseases of other aetiologies, is highlighted. Furthermore, future perspectives in the research regarding genetic susceptibility to fungal infection are depicted, particularly in what refers to the further clarification of the role of TLR polymorphisms in the normal function of the innate immune system and its impact in disease susceptibility.

CHAPTER 1

INTRODUCTION

1.1 INVASIVE FUNGAL INFECTIONS

Fungi are ubiquitous organisms present in the environment that are virtually unavoidable by humans. This omnipresence has led to the evolution of innate and acquired host defence antifungal mechanisms in multicellular organisms. Indeed, few fungal species regularly cause invasive disease in humans, and most of these act as opportunistic pathogens in individuals with specific immune defects; there are about 20 fungal species that cause >99% of human fungal infections, although about 600 different species have been reported as a cause of infection in man. Fungal infections are classified broadly into four groups: invasive, life-threatening infections (e.g. aspergillosis and candidiasis), mucosal infections, skin infections and allergic infections (e.g. asthma and chronic sinusitis).

1.1.1 EPIDEMIOLOGY

The availability of modern and sophisticated medical care to prolong and improve the lives of severely debilitated individuals has become increasingly common in the modern health care systems. Ironically, such medical advances have resulted in a group of patients more vulnerable to fungal infections. Such patients include those who receive immunosuppressive chemotherapy that produces prolonged neutropenia, such as bone-marrow or solid-organ transplant recipients (Fridkin and Jarvis, 1996). In fact, the number of life-threatening invasive fungal infections has risen dramatically over the last 20 years. Data collected in a 12-year study showed that the frequency of invasive fungal infections as judged after death by unselected autopsies had raised approximately 14 fold (Groll et al., 1996). More recently, this trend was confirmed in a hospital population-based study performed in several European countries for over 28 months. In particular, this study reported that invasive infection caused by *Candida* species ranged from 0.20 to 0.38 per 1,000 admissions and from 3.0 to 4.4 per 100,000 patient days (Tortorano et al., 2004).

Candida species are currently the fourth most common agent of all hospital-acquired bloodstream infection in the USA; such infections are increasingly significant causes of morbidity and with an estimated mortality of 25-38% (Wenzel and Edmond, 2001). Although sentinel surveillance studies in the 1980s indicated that the overall incidence of *Candida* bloodstream

infections increased among patients in hospital intensive care units (Banerjee et al., 1991), the incidence of infections caused by *C. albicans*, historically the most common agent in these infections, significantly decreased in this population in the 1990s (Trick et al., 2002). During the same period, a significant increase in *C. glabrata* bloodstream infections occurred (Trick et al., 2002). In fact, recent epidemiologic trends indicate a shift towards infections by non-*albicans Candida* species (Tortorano et al., 2004). In addition, *Aspergillus* species and previously uncommon fungi that often have little susceptibility to current antifungal agents are also becoming increasingly significant causes of fungal infection; the crude mortality from invasive aspergillosis is approximately 85% (Marr et al., 2002).

Several factors might be responsible for the emergency of non-*albicans Candida* species and other fungi. In addition to the abovementioned increase in the population of profoundly immunosuppressed patients who are at very high risk for developing infections, also the increased use of antifungal agents might select for non-*albicans* species that exhibit decreased susceptibility to these agents. For example, prophylactic use of fluconazole has been associated with the emergence of *C. krusei* and *C. glabrata* (Wingard et al., 1991; Wingard et al., 1993) and empiric or prophylactic use of amphotericin B has been linked with the emergence of *C. lusitaniae* and *C. glabrata* (Nguyen et al., 1996). Finally, increased recognition of the importance of non-*albicans Candida* species, not only because species distribution has changed in recent years, but also due to differences in susceptibility to antifungal agents, might lead to increased efforts to speciate *Candida* isolates in clinical microbiology laboratories.

1.1.2 DIAGNOSIS

Medical mycology has faced an enormous obstacle for decades: timely and accurate diagnosis of invasive fungal infections. Identification of *Candida* species has traditionally required isolation of the infecting organism from a normally sterile site, such as blood, peritoneal fluid or intravenous lines. In addition, these infections are difficult to diagnose because cultures are often negative or they become positive late in the disease; indeed, blood cultures were reported to be negative in up to 50% of all autopsy-proven cases of invasive candidiasis in such patients (Berenguer et al., 1993). If a culture is positive, accurate identification of the agent is laborious and time-consuming, relying on macroscopic and microscopic morphologic characteristics, biochemical tests, and serotyping. Regarding *Candida*, perhaps the most convenient and popular

methods for species identification are tests based on carbohydrate assimilation and/or enzyme detection which are commercially available in an assortment of different formats.

Physicians and microbiologists have recognized these limitations and have been intensively developing alternative diagnostic methods for decades. Some of these have been very successful, such as the *Cryptococcus* and *Histoplasma* antigen detection methods, which have become diagnostic standards due to their availability and diagnostic performance (Powderly et al., 1994; Wheat et al., 1986). Some, on the other hand, have been frustrating and laid to rest, such as *Candida* antibodies and metabolites (Reiss et al., 2000; Verweij et al., 1998).

Newer molecular methods employing polymerase chain reaction (PCR) technology offer the potential for more rapid and specific identification of fungal pathogens compared to traditional phenotypic methods, although not yet standardized or readily available in most clinical laboratories. Also, large clinical trials to determine the sensitivity and specificity of such molecular tests are nonexistent. Several PCR-based techniques have been developed using amplification of target DNA (Burgener-Kairuz et al., 1994; Crampin and Matthews, 1993; Fujita et al., 1995; Miyakawa et al., 1992). Fungal ribosomal genes are common DNA targets in PCR-based procedures for the identification of fungi at the species level. The highly variable sequences of internal transcribed spacer regions ITS1 and ITS2 flanked by the relatively conserved coding regions of 18S, 5.8S and 28S nuclear rRNA genes have been used in various PCR-based formats for the identification of clinically relevant yeasts (Chang et al., 2001; Elie et al., 1998; Fujita et al., 1995; Williams et al., 1995). There are now a multitude of techniques that include both quantitative and qualitative methods, real-time PCR, and combinations of PCR and enzyme-linked immunosorbent assay (ELISA), which can be carried out on blood and other human fluids (McMullan et al., 2008; Schabereiter-Gurtner et al., 2007).

The detection of fungal cell wall components, such as galactomannan and β-glucan is also very promising. The galactomannan assay for *Aspergillus* species has shown repeated good performance in a variety of settings and hosts, and widespread use is already ongoing (Meersseman et al., 2008; Steinbach et al., 2007; Woods et al., 2007). In addition, detection of β-glucan showed very good sensitivity and specificity, but more important, it showed an impressive negative predictive value (Odabasi et al., 2004).

1.1.3 THERAPY

For many years, the antifungal arsenal consisted only of amphotericin B desoxycholate (D-AmB) and 5-fluorocytosine (5-FC). However, side effects with the use of D-AmB are common, occurring in 50-90% of the cases, and are principally nephrotoxicity-related. Therapeutic alternatives only emerged with the clinical development of fluconazole and itraconazole in the late 1980s. Although inactive against filamentous fungi, fluconazole has been shown to be as effective as amphotericin B in the treatment of candidaemia in non-neutropenic patients (Phillips et al., 1997; Rex et al., 1994). However, the use of fluconazole may be inappropriate for the empirical treatment of suspected fungal infection, because prior exposure, as treatment or prophylaxis, is associated with the appearance of resistant strains of *Candida* species, and because of its lack of activity against moulds (Pappas et al., 2004). Itraconazole, on the other hand, has a wider spectrum than fluconazole, being active against yeasts and moulds, with some known exceptions (Johnson et al., 1998).

In the past 10 years, we have witnessed a significant expansion in antifungal drug research, which is reflected by the introduction of the lipid formulations of amphotericin B and the development of novel echinocandin derivatives (anidulafungin, caspofungin and micafungin) and improved antifungal triazoles (posaconazole, ravuconazole and voriconazole). Presently, amphotericin B, fluconazole and itraconazole are the cornerstones of treatment of invasive fungal infections. However, the use of other antifungal agents such as caspofungin is starting to look promising. Caspofungin has been shown to be as effective as amphotericin B, although better tolerated since its side effects are uncommon and usually mild (Mora-Duarte et al., 2002). At present, it is used mainly as a second-line agent in patients with life-threatening invasive *Candida* or *Aspergillus* infections unresponsive to first-line therapy.

Increased awareness among physicians, improved blood culture techniques and molecular diagnostic tools, together with the advent of high-resolution imaging techniques have had considerable impact on improving the clinical diagnosis of invasive fungal infections. In addition, major progress has been made in harmonizing disease definitions, in defining paradigms for antifungal intervention, and in designing and implementing clinical trials. Despite these advances, however, invasive fungal infections remain difficult to diagnose and to manage, and there is a continuing and urgent need for improved diagnosis, treatment, and prevention.

1.2 INNATE IMMUNITY TO FUNGAL PATHOGENS

Host defence mechanisms influence the manifestation and severity of fungal infections such that the clinical forms of the disease often depend on the immune response of the host. The innate immune response is focused mainly on physically eliminating the fungus, mostly by phagocytosis, while the adaptive response reacts specifically to, and neutralizes the pathogen and maintains a memory of it for safer and faster elimination on subsequent encounters. However, fungi also play a key role in the host-pathogen interaction. The pathogenesis of fungal infections involves several virulence factors that allow fungal survival and persistence in the host, eventually leading to tissue damage. Some virulence factors are of obvious importance, such as the structures through which fungi adhere to host tissues and the extracellular matrix, production of phospholipases, proteases and elastases that cause tissue damage and impairment of host defences, the ability to switch metabolic pathways that are required for intracellular survival, thermotolerance that allows dissemination to visceral organs, and the ability to survive in different forms and to reversibly switch from one to another during infection (Hogan et al., 1996; Latge, 2001).

1.2.1 THE INNATE IMMUNE SYSTEM

The immune system of vertebrates consists of two inter-related components, the innate and adaptive responses, which are jointly required for the resolution of most infections. The innate immune response, phylogenetically conserved and present in almost all multicellular organisms, is the first line of host defence and is responsible for immediately recognizing and countering microbial invasion (Hoffmann et al., 1999). This arm of the immune system is comprised mainly of phagocytic cells such as macrophages and neutrophils, which can ingest and kill the invading pathogens. These cells can also instruct the adaptive immune response about the nature of the pathogenic challenge through cytokine and chemokine production, expression of costimulatory molecules and presentation of microbial antigens to lymphocytes leading to the development of a highly specific immune response that is tailored to combat the individual microorganism.

The specificity of the adaptive immune response relies on the recognition of peptide antigens using antigen receptors expressed on the surface of lymphocytes. In order to provide specific recognition of a wide range of potential antigens, lymphocytes are able to rearrange their receptor genes through random somatic mutation generating a selection of receptors that best recognize the microbial antigens. Engagement of these receptors by the cognate antigen triggers clonal expansion of the lymphocyte and further production of antigen-specific antibodies. By contrast, the innate immune response is based on a limited number of evolutionarily conserved germline-encoded receptors, the pattern recognition receptors (PRRs), which recognize highly conserved microbial structures, enabling the host to rapidly recognize a broad range of pathogens (Janeway and Medzhitov, 2002). These microbial components, known as pathogen-associated molecular patterns (PAMPs), are essential for the survival of the microorganisms and are therefore difficult to alter. Furthermore, innate immunity also constantly monitors host internal environment to detect any endogenous changes which might be caused by tissue injury, apoptosis, viral infection and/or tumour formation (Medzhitov and Janeway, 2002).

PRRs can be broadly categorized into three groups based on their cellular localization – serum/tissue fluid, membrane or cytoplasm – and further subdivided into related families based on structure and/or function. Leukocytes use these receptors to recognize pathogens either directly or indirectly, whereby distinct opsonic receptors recognize PRR-coated pathogens. Recognition also occurs in vacuoles after microbe uptake, or in the cytoplasm, which is required for the detection of intracellular pathogens. During infection *in vivo*, microbial recognition is likely to occur through multiple interactions at multiple sites and to involve many receptors.

Following recognition, some PRRs promote microbial uptake through the actin-dependent process of phagocytosis. Microbial killing then occurs in the resultant vacuole (known as the phagosome) and is achieved through various mechanisms, such as lowering of the phagosomal pH, production of reactive oxygen intermediates (ROIs) and release of hydrolytic enzymes and toxic metabolites into the phagosome. Microbial recognition and uptake also leads to the induction of an inflammatory response – including the production of cytokines and chemokines – which activates and recruits other cells to the site of infection, leading ultimately to the initiation of the adaptive arm of the immune response.

1.2.2 PATHOGEN RECOGNITION BY TOLL-LIKE RECEPTORS

The field of innate immunity was guided to its present era of accelerated development mostly due to the discovery of a class of membrane PRRs known as Toll-like receptors (TLRs). In 1996, the *Drosophila* protein Toll, originally identified as a transmembrane receptor required for the establishment of dorso-ventral polarity in the developing fly embryos (Belvin and Anderson, 1996; Hashimoto et al., 1988), was shown to be required for flies to mount effective antifungal responses (Lemaitre et al., 1996).

In the *Drosophila*, stimulation of Toll by the secreted Spätzle factor, a ligand of Toll, activates the cytoplasmic serine/threonine kinase Pelle via the adaptor protein Tube. Spätzle is a polypeptide precursor that requires proteolytic cleavage by serine proteases for activation. Activation of Pelle promotes degradation of the ankyrin-repeat protein Cactus, a homologue of mammalian inhibitor of nuclear factor-kappa B (NF-κB) IκB which associates with the NF-κB family transcription factor Dorsal in the cytoplasm. Once Cactus is degraded in response to the Toll-mediated signal, Dorsal is free to translocate to the nucleus, where it regulates transcription of specific target genes (Belvin and Anderson, 1996).

The signalling pathway of *Drosophila* Toll shows remarkable similarity to the mammalian interleukin (IL)-1 pathway, which leads to the activation of NF-κB, a transcription factor responsible for many aspects of inflammatory and immune responses. Indeed, the cytoplasmic domains of *Drosophila* Toll and the mammalian IL-1 receptor are highly conserved and referred to as the Toll/IL-1 receptor (TIR) domain. In addition to the presence of homologous cytoplasmic TIR domains, both receptors can induce NF-κB activation and signal through homologous protein kinases – Pelle and IL-1 receptor-associated kinase (IRAK) (Anderson, 2000; Belvin and Anderson, 1996). Based on this similarity, it was proposed that the Toll-mediated pathway could be involved in regulating immune responses (Belvin and Anderson, 1996). This was clearly demonstrated in a study involving mutant flies lacking individual components of the Toll-mediated pathway, i.e., Toll, Spätzle, Tube or Pelle (Lemaitre et al., 1996). The mutant flies demonstrated a striking defect in immune responses, being highly susceptible to fungal infection owing to a lack of expression of the antifungal peptide drosomycin.

A year after the discovery of the role of the *Drosophila* Toll in the host defence against fungal infection, a mammalian homologue, now known as TLR4, was identified (Medzhitov et al., 1997). Subsequently, a family of proteins structurally related to *Drosophila* Toll was also

identified. The human TLR family is known to consist of at least 12 members that differ from each other in ligand specificities, expression patterns and presumably, in the target genes they can induce (Chuang and Ulevitch, 2000; Du et al., 2000; Medzhitov et al., 1997; Rock et al., 1998; Takeuchi et al., 1999; Zhang et al., 2004). Besides their cytoplasmic TIR domain, these receptors are structurally characterized also by the presence of a leucine-rich repeat (LRR) domain in their extracellular portion, composed of 19-25 tandem LRR motifs, each of which is 24-29 amino acids in length. It was imagined that the LRR domain of the TLR would form a horseshoe structure with the ligand binding to the concave surface. However, the three-dimensional structure of the human TLR3 has recently been elucidated, and its structure suggests a somewhat different model in which negatively charged dsRNA is more likely to bind the outside convex surface of TLR3 (Choe et al., 2005).

TLRs are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific subsets of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells. Expression of TLRs is not static but is rather rapidly modulated in response to pathogens, a variety of cytokines and environmental stresses. Furthermore, TLRs may be expressed either extra- or intracellularly; while certain TLRs (TLR1, -2, -4, -5 and -6) are expressed on the cell surface, others (TLR3, -7, -8 and -9) are found almost exclusively in intracellular compartments such as endosomes, and their ligands, mainly nucleic acids, require internalization to the endosome before signalling is possible.

The engagement of TLRs by microbial components triggers the activation of signalling cascades, leading to the induction of genes involved in antimicrobial host defence (Akira and Takeda, 2004). After ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR domain-containing adaptor proteins to the cytoplasmic portion of the TLRs through homophilic interaction of their TIR domains. There are four adaptor molecules, namely myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TIR domain-containing adaptor protein inducing interferon (IFN)-β (TRIF)/TIR domain-containing molecule 1 (TICAM1) (Oshiumi et al., 2003) and TRIF-related adaptor molecule (TRAM). The differential responses mediated by distinct TLR ligands can be explained in part by the selective usage of these adaptor molecules. MyD88 and TRIF are responsible for the activation of distinct signalling pathways, leading to the production of proinflammatory cytokines and type I IFN, respectively (Figure 1).

MyD88 is critical for the signalling from all TLRs except TLR3. Upon TLR stimulation, MyD88 associates with their TIR domains and recruits IRAK-4 and IRAK-1 through a homophilic interaction of their death domains. In TLR2 and TLR4 signalling, another adaptor, TIRAP/MAL, is required for recruiting MyD88 to the receptor (Fitzgerald et al., 2001; Horng et al., 2002; Yamamoto et al., 2002). After IRAK-1 associates with MyD88, it is phosphorylated by the activated IRAK-4 and subsequently associates with tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), which acts as an ubiquitin ligase (Li et al., 2002). TRAF6, together with an ubiquitination enzyme complex, catalyzes the formation of a polyubiquitin chain on TRAF6 itself and on inhibitor of NF-κB kinase complex (IKK)-γ/NF-κB essential modulator (NEMO) (Deng et al., 2000). The ubiquitinated TRAF6 then associates with a complex composed of transforming growth factor (TGF)-β-activated kinase 1 (TAK1) and the TAK1-binding proteins 1 and 2 (TAB1 and TAB2) (Wang et al., 2001a). TAK1 is able to activate the inhibitor of the IKK. IKK-mediated phosphorylation of IκB leads to its degradation, allowing NF-κB to translocate to the nucleus and promote the transcription of multiple proinflammatory genes. In addition, TAK1 can also activate mitogen-activated protein (MAP) kinases, such as c-Jun N-terminal kinases (JNKs) and p38, leading to the activation of activator protein 1 (AP-1) to regulate the expression of proinflammatory cytokine genes (Sato et al., 2005). In addition to NF-κB and AP-1, the transcription factor IFN regulatory factor 5 (IRF-5) also regulates the expression of cytokine genes, binding to potential IFN-stimulated response element (ISRE) motifs present in the promoter regions of these genes (Takaoka et al., 2005).

Stimulation with TLR3, -4, -7, -8 and -9 ligands, but not TLR2 ligands, induces type I IFN production via a MyD88-independent pathway in addition to proinflammatory signals. The activity of this pathway leads to DC maturation, expression of costimulatory molecules and type I IFN secretion, inducing an antiviral state in various cells in addition to modulating the development of adaptive immunity (Kaisho and Akira, 2001). This MyD88-independent pathway is initiated by another TIR domain-containing adaptor, TRIF (Hoebe et al., 2003; Yamamoto et al., 2003). TRIF recruitment to the TIR domain of TLR4 is specifically coordinated by TRAM (Fitzgerald et al., 2003b; Yamamoto et al., 2003). TRIF then interacts with receptor-interacting protein 1 (RIP1), a kinase responsible for the activation of NF-κB (Meylan et al., 2004). On the other hand, TRIF is also able to activate the TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) via TRAF3 (Hacker et al., 2006; Oganesyan et al., 2006). TBK1 is known to comprise a family of inducible IκB kinases that are able to directly phosphorylate IRF-3 and/or IRF-7

(Fitzgerald et al., 2003a; Sharma et al., 2003). Phosphorylated IRF-3 and IRF-7 form homodimers, translocate into the nucleus, and bind to the ISRE motifs, resulting in the expression of a set of IFN-inducible genes.

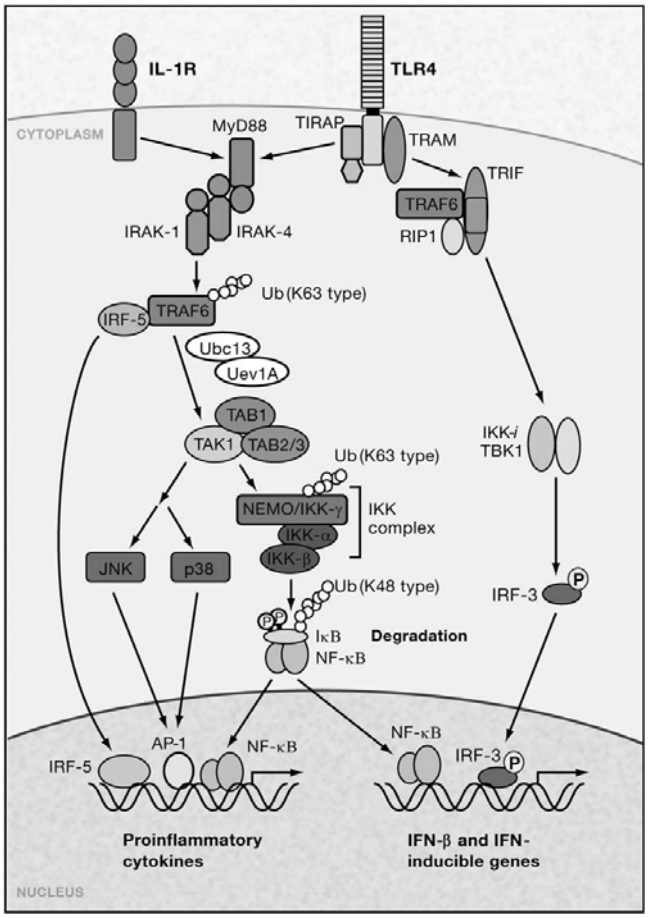

Figure 1. The TLR signalling pathway. Adapted from Akira et al., 2006.

1.2.3 TOLL-LIKE RECEPTORS IN ANTIFUNGAL INNATE IMMUNITY

The observation that Toll-deficient *Drosophila* were highly susceptible to fungal infection led to the assumption that mammalian TLRs also participated in antifungal immunity. In fact, several PAMPs located in the cell wall or on the cell surface of fungi are recognized by TLR2 or TLR4. The T helper (Th) 1 response is critical in protection against fungi; although TLR-mediated signals mostly induce Th1-directed responses, activation of TLR2 is less inflammatory and

favours the development of the Th2 response through the induction of IL-10 (Agrawal et al., 2003). Indeed, *in vivo* infection experiments using mutant mice suggest differential roles of TLR2 and TLR4 in fungal infection. TLR4-deficient mice showed increased susceptibility to disseminated *Candida* infection, whereas TLR2-deficient mice showed increased resistance (Netea et al., 2004). The higher susceptibility displayed by $TLR4^{-/-}$ mice was mediated through decreased release of the chemokines keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP2), and through impaired recruitment of neutrophils at the site of infection. In contrast, production of proinflammatory cytokines such as TNF and IL-1β were only marginally influenced, whereas the candidal killing capacity of TLR4-defective phagocytes was normal. On the other hand, the TLR2-mediated anti-inflammatory signals leading to IL-10 release and generation of positive cluster of differentiation 4 and 25 ($CD4^{+}$ $CD25^{+}$) T regulatory cells were shown to be responsible for the increased resistance of TLR2-deficient mice to disseminated *Candida* infection. These data demonstrate that *C. albicans* is able to use TLR2 recognition and signalling to induce immunosuppression through IL-10 production, thereby escaping host defence.

A similar escape mechanism from the host defence is also observed in *A. fumigatus* infection. TLR2 and TLR4 have been implicated in host defence against *A. fumigatus* and *A. niger*, a non-pathogenic *Aspergillus* species. Germination of conidial forms is an important mechanism for initiating and establishing an infection, and both *Aspergillus* conidia and hyphae stimulate cytokine production via TLR2 in mice (Mambula et al., 2002), whereas only conidia are capable of stimulating murine macrophages via TLR4 (Netea et al., 2003). Thus, the phenotypic switch from conidia to hyphae, resulting in the release of IL-10 through TLR2-dependent mechanisms, impairs the cellular immune response necessary for the *Aspergillus* clearance. This indicates that activation of specific cell surface receptors during germination may allow *Aspergillus* to counteract host defences, contributing to its survival. In addition, *Aspergillus* conidia and hyphae induce NF-κB translocation and release of TNF-α in a TLR2- and TLR4-dependent manner, which is associated with chemokine production and influx of neutrophils and monocytes into infected tissues. Mice lacking functional chemokine receptor 1 (CCR1) are much less efficient at blocking tissue invasion and have increased susceptibility to infection (Gao et al., 1997). In the same way, neutrophil recruitment is severely impaired in mice lacking both functional TLR2 and TLR4, but is less impaired in single TLR2- or TLR4-deficient mice, suggesting

that both receptors are required for an optimal immune response to *Aspergillus* (Meier et al., 2003).

In addition to TLR2 and TLR4, the role of TLR9 in the host defence against *Candida* was also investigated (Bellocchio et al., 2004a). While no increased susceptibility of TLR9$^{-/-}$ mice to disseminated candidiasis has been observed, the fungal burden in the organs of deficient animals tended to be lower than that in control mice. However, in contrast to TLR2$^{-/-}$ mice, TLR9$^{-/-}$ animals produced less IL-12 and more IL-10 and IL-4 than control mice. It is not know why this shift towards an anti-inflammatory cytokine profile, known to be deleterious for the anticandidal host defence, did not result in such effect on the outcome of the infection.

With regard to the specific signalling pathways activated by these fungi, responses to *A. fumigatus* have been reported to occur in a MyD88-independent manner (Marr et al., 2003). By contrast, mediation of macrophage phagocytosis and killing of *C. albicans* requires the involvement of MyD88, suggesting that murine macrophages might use different pathways that are specific to different types of pathogenic fungi. In addition, the MyD88-dependent signal transduction pathway is required for adaptive Th1 cell-mediated resistance to both *C. albicans* and *A. fumigatus* (Bellocchio et al., 2004a). It has also been demonstrated that dectin-1, a non-classical C-type lectin expressed mainly in myeloid cells and that binds β-glucans, can collaborate with TLR2 in response to yeast to elicit a strong inflammatory response via recruitment of the protein tyrosine kinase Syk (Gantner et al., 2003; Rogers et al., 2005; Underhill et al., 2005). However, although β-glucan is presented during *C. albicans* yeast growth, it is not presented during filamentous growth. As a consequence, dectin-1-mediated antimicrobial defences are not effective against filaments, which may explain why they are more virulent.

1.2.4 ANTIFUNGAL EFFECTOR MECHANISMS

The antifungal effector functions of phagocytes include killing and growth inhibition of fungi, as well as pathways to oppose fungal invasion, including effects on dimorphism and phenotypic switching. The optimal restriction of fungal growth occurs through a combination of oxidative and complementary non-oxidative mechanisms, the latter consisting of degranulation and intra- or extracellular release of effector molecules, defensins and neutrophil cationic peptides, and iron sequestration (Romani, 2004). Enzymes such as the nicotinamide adenine

dinucleotide phosphate (NADPH) oxidase and the inducible nitric oxide synthase (iNOS) initiate the oxidative pathways known as respiratory burst, in which toxic ROIs are produced, the nature of which depends on the pathogens and the type of phagocytic cell. ROIs damage fungi by inducing protein modifications, nucleic acid breaks and lipid peroxidation (Mansour and Levitz, 2002). In retaliation, fungi have evolved strategies to selectively inhibit the respiratory burst through production of ROI scavengers, such as catalase, mannitol and melanin (Hamilton and Holdom, 1999). Some fungi have also developed various mechanisms or putative virulence factors to evade phagocytosis and survive inside macrophages (Brandhorst et al., 1999; Ibata-Ombetta et al., 2003; Tucker and Casadevall, 2002; Woods, 2003). In fact, macrophages serve as a protected environment in which the dimorphic fungi, such as *Histoplasma capsulatum*, a classical example of a successful intracellular fungal pathogen, multiplies and disseminates from the lungs to other organs (Woods, 2003).

Several components of the innate immune response, such as complement, collectins and antibodies, are essential in promoting opsonization and recognition of fungi by various receptors. In fact, humoral factors are known to be essential in the innate immune response to *Aspergillus*. Resting conidia, germinating conidia and hyphae are potent activators of the complement cascade and induce deposition of complement components upon the fungal surface. In the alveolar fluid, pulmonary surfactant protein A (SP-A) and D (SP-D) enhance chemotaxis, binding, phagocytosis and oxidative killing of *Aspergillus* (Madan et al., 1997). These C-type lectins also agglutinate *Aspergillus* conidia, thereby immobilizing the pathogen. These immune mechanisms are also present in the host response to *C. albicans*. The fungus activates complement by the classical and alternative pathways with deposition on the cell fungal surface, facilitating the recruitment of phagocytes to infected tissues and enhancing their anticandidal activity. However, despite the importance of these humoral factors in experimental systems, the predisposing factor for the vast majority of patients with invasive disease appears to be phagocytic dysfunction rather than defects in humoral immunity (Andrews and Sullivan, 2003).

Antibodies contribute to the activation of the complement system by fungi (Kozel, 1996) and complement is essential for antibody-mediated protection (Han et al., 2001). Studies with *Cryptococcus neoformans* have shown that the high levels of carbon dioxide in the lungs favour capsule formation, impairing phagocytosis in the absence of capsular antibodies (Syme et al., 1999). These antibodies can alter the conformation of the capsule and so directly favour binding and phagocytosis of the yeasts (Taborda and Casadevall, 2002). However, antibodies have

disparate biological effects in fungal infections, with both protective and non-protective antibodies being induced during infection (Bromuro et al., 2002). Complement, antibodies and collectins not only fulfil the requirement of a first line of defence against fungi, but also have an impact on the inflammatory and adaptive immune responses through several mechanisms, including regulation of cytokine secretion and expression of costimulatory molecules by phagocytes (Sato et al., 2003; Vecchiarelli et al., 2002).

Cell surface and secreted PRRs mediate the development of appropriate Th responses. In this regard, pentraxin 3 (PTX3) has been shown to be required for the prompt handling of *Aspergillus* conidia by alveolar macrophages. In fact, PTX3-deficient mice are susceptible to invasive pulmonary aspergillosis due to defective recognition of conidia by alveolar macrophages and DCs, and lack of development of appropriate Th1 responses (Garlanda et al., 2002). These deleterious effects can be reversed by treatment with purified PTX3, which restores both phagocytic and conidiocidal activities (Garlanda et al., 2002).

1.3 GENETIC SUSCEPTIBILITY TO INFECTIOUS DISEASES

During the past century, differences in human susceptibility to several infectious diseases have been described, the best known example being malaria. Individuals who are heterozygous for haemoglobin S (HbS) are protected against infection by *Plasmodium falciparum*, while those homozygous for HbS have sickle cell anaemia (Allison, 1954). Although malaria provides an example of a genetic variant resulting in decreased susceptibility to an infectious disease, there is also evidence that genetic variants can lead to an increased occurrence of infections. In fact, a study comparing the causes of death among adopted children with those of either their biological or adoptive parents led to the conclusion that children had an increased risk of death resulting from infections when their biological parents also prematurely died from the same cause (Sorensen et al., 1988). This study, which effectively separated genetic and environmental confounders, confirmed the substantial genetic effect involved in susceptibility to infection.

1.3.1 HUMAN GENETICS: EXPLORING SUSCEPTIBILITY TO DISEASE

Although humans are identical at most of the 3 billion base pairs in their genome, inter-individual variation is present in approximately 0.01% of the genome (Goldstein and Cavalleri, 2005). Since the mutation rate in mammalian genomes is low (10^{-9} per bp per year), most inter-individual variations are inherited. The most common type of human genetic variation is the single nucleotide polymorphism (SNP), in which two alternative bases occur at appreciable frequency (>1%) within a population. Another type of genetic mutation is the variable number of tandem repeats (VNTR), consisting of repeats of sequences ranging from a single to thousands of base pairs (Ellegren, 2004). Since the number of repeats varies among individuals, VNTRs have been widely used as genetic markers. Once markers have been typed, two main approaches can be used to analyse them: single marker analysis or haplotype analysis, the latter referring to the arrangement of two or more alleles on the same chromosome. In addition, certain SNPs or VNTR alleles, or both, may be linked together so that non-functional polymorphisms can be used as genetic markers of functionally important mutations.

Only 1.5% of SNPs are thought to be located in the coding region of a gene, with the functions of nearly all SNPs that are located outside gene coding or regulatory regions being unknown. These genetic variations, however, are not randomly distributed within the genome, but rather depend on the particular genomic region, as well as on selective pressure (Zhang and Hewitt, 2003). Hence, it is reasonable to speculate that genes of the innate immune system may represent a major source of variability regarding susceptibility to infectious diseases. Although natural immunity ensures survival of the species as a whole, individuals themselves are not likely to be immunocompetent to all pathogens, and individual differences in susceptibility to specific pathogens are quite common.

The traditional approach to study a genetic trait is to narrow down the genetic region of interest by use of linkage analysis, followed by fine mapping and association studies (Bochud et al., 2007). Linkage and association studies are based on the same underlying principle; once a mutation occurs on a particular chromosome, it is subsequently transmitted to offspring together with nearby loci. This association is broken down at each successive generation by recombination. When two loci are close enough on the same chromosome that their alleles cosegregate when passed on to the next generation, the two loci are said to be in linkage disequilibrium.

Whereas linkage and association studies can both be carried out in families, only association studies can be performed in unrelated cases and controls. Thus, association studies are able to capitalize on all meiotic recombination events in a population, rather than only those in the families studied; because of this, association signals are localized to small regions of the chromosome containing only a single to a few genes, enabling rapid detection of the actual disease susceptibility gene. Also, association studies allow the identification of disease genes with only modest increases in risk, a severe limitation in linkage studies. Due to these advantages, association studies can identify multiple interacting disease genes and their respective pathways, providing a comprehensive understanding of the aetiology of disease. On the other hand, the power to detect association between genetic variation and disease is a function of several factors, including the frequency of the risk allele or genotype, the relative risk conferred by the disease-associated allele or genotype, the correlation between the genotyped marker and the risk allele, sample size, disease prevalence and heterogeneity, and genetic heterogeneity of the sampled population.

1.3.2 Polymorphisms in Toll-like receptors

Several genetic variants have been described within the pattern recognition molecules involved in innate immunity, affecting their functional activity. A major example is that of mannose-binding lectin (MBL) deficiency, one of the most common human immunodeficiencies. MBL is a member of the collectin family, which also includes SP-A and SP-D (Epstein et al., 1996; Holmskov, 2000). Since it was first reported, MBL deficiency has been consistently associated with increased susceptibility to many infectious diseases, particularly when adaptive immunity is compromised (e.g., in early childhood (Koch et al., 2001; Summerfield et al., 1997) or following chemotherapy (Eisen and Minchinton, 2003; Peterslund et al., 2001)). Five functional SNPs exist within the *MBL2* gene, each affecting serum levels of the protein. Three polymorphisms are present at codons 52, 54 and 57 encoding for variant alleles D, B, and C, respectively, which dramatically reduce functional MBL levels by impairing the assembly of MBL monomers into functional oligomers (Garred et al., 2003). Two additional SNPs at positions -551 (H/L), and -221 (X/Y) in the 5' flanking region of the *MBL2* gene affect transcriptional activity of the basal-promoter complex and reduce levels of circulating MBL (Madsen et al., 1995). Of these, two promoter haplotypes, HY, which is associated with high levels of MBL, and LX, which

is associated with low MBL levels, appear to be the most important. Through the combination of structural and promoter polymorphisms, MBL concentrations can vary considerably in apparently healthy individuals (Steffensen et al., 2000).

The impact of genetic variants in the functional activity of innate immunity components was further elucidated by the discovery of two cosegregating polymorphisms of the human *TLR4* gene – Asp299Gly and Thr399Ile. These SNPs were described to be present at a substantially higher proportion among individuals hyporesponsive to inhaled lipopolysaccharide (LPS) (Arbour et al., 2000). Epithelial cells derived from these individuals were unable to mediate LPS signalling *in vitro*, both in the homozygous and heterozygous state. Moreover, similar findings were observed with alveolar macrophages, with the wild-type allele being able to reverse the phenotype. Finally, THP-I cells transfected with the mutated human *TLR4* gene also displayed lower responses to administered LPS. Because these SNPs are located within the extracellular domain of TLR4, the impact is most likely caused by impairment in ligand recognition. However, functional studies indicated that the Asp299Gly genotype might have a greater functional impact compared with the Thr399Ile genotype (Arbour et al., 2000).

This report was followed by a series of studies investigating the potential impact of these SNPs on the incidence and course of infectious diseases (Cook et al., 2004). One of them confirmed an association of these SNPs with the incidence of septic shock during infections with Gram-negative bacteria (Lorenz et al., 2002b). These data were, in part, supported by studies investigating the distribution of Gram-negative pathogens in critically ill patients in a surgical setting (Agnese et al., 2002). In contrast, two studies investigating the influence of the *TLR4* SNPs on the incidence of sepsis after surgery (Lorenz et al., 2001b) and severity of inflammatory response syndrome (Child et al., 2003) have failed to detect a significant correlation. One problem may be that the aetiology of sepsis is heterogeneous and that *TLR4* SNPs would only be predicted to alter susceptibility to Gram-negative infections. In addition, several rare *TLR4* coding variants were markedly over-represented in patients with systemic meningococcal infections caused by the Gram-negative organism *Neisseria meningiditis*, supporting the notion that rare, rather than common, variants of *TLR4* may be associated with susceptibility to infectious disease (Smirnova et al., 2003).

The Asp299Gly polymorphism has also been correlated with a reduced risk for carotid artery atherosclerosis (Kiechl et al., 2002) and acute coronary events (Ameziane et al., 2003; Boekholdt et al., 2003). Individuals with this polymorphism have lower concentrations of

circulating proinflammatory cytokines such as IL-6, fibrinogen and soluble vascular cell adhesion molecule 1 (VCAM-1). These molecules have well described functions in inflammation, which in turn is associated with atherosclerotic progression, plaque rupture and consequent vessel occlusion. The finding that the heat shock protein HSP60 from *Chlamydia pneumonia* can activate TLR4 signalling (Sasu et al., 2001) might be of particular relevance to vascular disease because this protein has been found in atherosclerotic lesions. Recognition of HSP60 by human TLR4 might exacerbate the inflammatory component of atherosclerosis in individuals harbouring *C. pneumonia*, whereas those with the Asp299Gly polymorphism might be partially protected by decreased recognition/signalling. In addition, the association between TLR4 function and atherosclerosis is consistent with findings showing that TLR4 mRNA and protein are more abundant in plaques in atherosclerotic lesions than in unaffected vessels (Xu et al., 2001).

A non-synonymous SNP within a conserved part of the C-terminal region of human TLR2 (Arg753Gln) was also reported (Lorenz et al., 2000). As shown for Asp299Gly and Thr399Ile, this polymorphism led to a decreased activation of transfected HEK293 cells by TLR2 ligands *in vitro* (Lorenz et al., 2000). Arg753Gln has been associated with susceptibility to tuberculosis (Ogus et al., 2004). Interestingly, it also appeared to protect from the development of late stage Lyme disease, perhaps due to a reduced signalling via TLR2 (Schroder et al., 2005). In contrast, there was no apparent association between this SNP and disease or mortality attributable to *S. aureus* infection (Moore et al., 2004).

Besides TLR4 and TLR2, other TLRs were studied with regard to their genetic variation and association to disease susceptibility. Two variants of human TLR9 were identified within the promoter region, as well as a number of rare non-synonymous SNPs within the coding sequence (Lazarus et al., 2003). An association of one of the promoter SNPs – T-1237C – with asthma was reported, whereas no significant results were found regarding the other variants. In addition, a role for this polymorphism in Crohn's disease was also reported (Torok et al., 2004). Previous suggestions were made that TLR9 signalling mediated the anti-inflammatory effects of probiotics in murine experimental colitis model (Rachmilewitz et al., 2004), thus differences in *TLR9* expression could very well be involved in Crohn's disease pathogenesis. More recently, the same SNP was also reported to be involved in the susceptibility to atopic eczema and alterations in *TLR9* expression were considered to underlie this putative association (Novak et al., 2007).

A common dominant stop codon occurring within the human *TLR5* gene (392STOP) has also been described (Hawn et al., 2003), abolishing flagellin-induced signal transduction, thus

underlying increased susceptibility to pneumonia caused by *Legionella pneumophila*, a flagellated bacterium.

In addition to polymorphisms in TLRs, genetic variants have also been identified within molecules involved in TLR signalling, namely CD14. Specifically, two SNPs within the promoter region of CD14 were reported, and there is evidence that one of them is related to the incidence and mortality of septic shock (Schroder et al., 2003). Several reports also emphasized the importance of these SNPs for cardiovascular diseases as well, possibly reflecting the inflammatory nature of atherosclerosis pathology (Arroyo-Espliguero et al., 2004).

1.3.3 SUSCEPTIBILITY TO FUNGAL INFECTIONS

During the past two decades, invasive fungal infections have emerged as a major threat to immunocompromised hosts. Prolonged neutropenia, T cell depletion, graft-versus-host disease (GvHD) and the administration of immunosuppressive agents all contribute to the high incidence of these infections (Maury et al., 2001). Both host and pathogen factors determine the clinical outcome of invasive fungal infections and, whereas some variation may be attributable to fungi and environmental variables, it is probable that the host genetic background also plays a significant role. Genetic analysis of innate immune genes may identify a subset of patients most vulnerable to fungal infections and this ability to predict susceptibility to specific infections can be directed towards the design of novel therapeutic approaches to protect these patients.

Although the immunological status of the host is probably the main determinant of the clinical presentation of fungal infections, very little is known about the genetic susceptibility to these infections. Recent evidence has focused on proteins such as SP and MBL, which have been identified as key players that strengthen the innate immune response by interacting with pathogens and with immune effector cells (van de Wetering et al., 2004). More specifically, an important role for SP-A and MBL in host defence against *A. fumigatus* was described (Madan et al., 2005). In fact, the presence of the common mutation in codon 52 of MBL (variant D) has been shown to significantly associate with chronic cavitary pulmonary aspergillosis (CCPA) (Vaid et al., 2007), which is in accordance to earlier findings that reported a significant correlation of the same mutation with the other form of chronic pulmonary aspergillosis, CNPA (Crosdale et al., 2001). Unlike CCPA patients, no significant association between variant D of MBL and susceptibility to allergic bronchopulmonary aspergillosis (ABPA) was observed. However, a

polymorphism in the intronic region of MBL has been shown to associate with ABPA, and elevated plasma MBL levels and activity, as well as eosinophilia were suggested as the underlying mechanisms of susceptibility to this form of allergic aspergillosis (Kaur et al., 2006).

Allele variants in *SP-A2*, one of the genes encoding for functional SP-A, have been associated with increased susceptibility to both ABPA and CCPA (Vaid et al., 2007). One of those variants, a non-synonymous SNP, results in the substitution of an alanine residue for a proline residue in the collagen domain of SP-A2, in which proline residues provide structural stability to the triple helical collagen structures. Although the precise effect of this change on functionality of SP-A is not yet known, patients with ABPA carrying the mutant allele showed a marked increase in total immunoglobulin E (IgE) antibodies and peripheral eosinophilia as well as decreased lung performance (Saxena et al., 2003). The occurrence of distinct genotype combinations of *SP-A2* and *MBL2* in CCPA and ABPA patients suggests that these combinations, along with other genetic factors, may confer distinct immune status to the host and may thus be partially responsible for the pathogenesis of these diverse clinical entities caused by the same fungus.

The dominance of either Th1 or Th2 pathways directly correlates with the outcome and severity of fungal infections. Polymorphisms in cytokine genes have been associated with CCPA and could explain the devastating consequences of the disease in otherwise healthy individuals without any obvious immune defect. Sambatakou and colleagues showed that the presence of a particular allele variant in the gene encoding for IL-15 was found to be common among CCPA patients, leading them to produce high levels of the cytokine (Sambatakou et al., 2006). IL-15 promotes a non-protective Th2 response, thus rendering these individuals more susceptible to aspergillosis. In contrast, a mutation in the promoter region of the gene encoding for TNF-α, known to underlie elevated levels of the cytokine, is less common in CCPA patients (Sambatakou et al., 2006). Accordingly, *in vitro* studies have demonstrated that TNF-α enhances specific phagocytic activity against conidia by pulmonary alveolar macrophages and augments the neutrophil damage of *Aspergillus* hyphae (Roilides et al., 1998b).

The inheritance of a specific allele variant in the promoter region of the gene encoding for IL-10 was also associated with the development of CCPA (Sambatakou et al., 2006). The presence of this variant correlates with reduced IL-10 production by lymphocytes, suggesting that the regulatory role of IL-10 upon pulmonary immune responses and in suppressing acute inflammation seems to be crucial to the development of CCPA. In contrast, non-CCPA aspergillosis appears to associate with genotypes leading to high levels of IL-10 (Sambatakou et

al., 2006). Accordingly, a significant relationship between the high IL-10 production genotype and both colonization with *A. fumigatus* and ABPA in patients with cystic fibrosis was also reported (Brouard et al., 2005). In addition, elevated IL-10 concentrations have been reported to associate with the development of invasive aspergillosis (IA) (Roilides et al., 2001).

Since allogeneic haematopoietic stem cell transplantation (HSCT) dramatically impairs various layers of the innate immune response, subtle defects in immune recognition could become clinically significant. In fact, in healthy individuals, the redundancy of the different innate immune pathways could camouflage a subtle defect. A specific IL-10 promoter haplotype has been shown to have a protective impact upon the development of IA after allogeneic HSCT regardless of human leukocyte antigen (HLA) disparity or GvHD, although no correlation with IL-10 serum levels was studied (Seo et al., 2005). In accordance, Sainz and colleagues also reported a protective role for the presence of a specific genotype of the IL-10 promoter in the pathogenesis of IA after allogeneic HSCT, suggesting that a differential production of IL-10 may alter the risk for IA in haematological patients (Sainz et al., 2007a).

The primary effector cells of the innate immune response against *A. fumigatus* are alveolar macrophages that can induce production of inflammatory cytokines such as TNF-α (Taramelli et al., 1996). In addition to TNF-α, lymphotoxin (LT)-α is also considered a pivotal mediator of inflammatory responses to infections with fungi (Bazzoni and Beutler, 1996). However, polymorphisms in the genes encoding for these cytokines were not associated with the development of IA, results consistent with the similarity in plasma cytokine levels between the groups of patients analyzed (Sainz et al., 2007b). Also, no correlation was found between IA and polymorphisms in the TNF receptor type 2 (*TNFR2*) gene, known to disrupt effective signalling. In contrast, a polymorphic site in the promoter region of *TNFR2* was shown to predispose to IA (Sainz et al., 2007b). It is possible that this variant sequence in the promoter region of *TNFR2* decreases its expression, thus explaining the increased susceptibility to IA. However, functional studies are yet required to fully understand the impact of this VNTR in the pathogenesis of IA.

In addition to cytokine genes, polymorphisms within PRRs were also correlated with increased susceptibility to IA after allogeneic HSCT. In fact, polymorphisms in MBL-associated serine protease 2 (MASP-2), known to lead to deficiencies in the MBL pathway, were shown to be predictive factors for IA after allogeneic HSCT (Granell et al., 2006). Additionally, Kesh and colleagues have shown that a particular polymorphism in *TLR1* (Arg80Thr) or the simultaneous occurrence of SNPs in both *TLR1* and *TLR6* (Asn248Ser and Ser249Pro, respectively)

predisposed to IA after allogeneic HSCT (Kesh et al., 2005). Interestingly, in the same study, no association with the clinically relevant *TLR4* polymorphisms was observed.

Because TLR4 has been implicated in the host defence against *Candida* species (Netea et al., 2002), common genetic variants within this receptor were proposed to underlie increased susceptibility to *Candida* infections. In fact, Van der Graaf and colleagues demonstrated that Asp299Gly and the co-segregated Thr399Ile polymorphisms of *TLR4* were associated with an increased risk for *Candida* bloodstream infection (Van der Graaf et al., 2006). In addition, an increased IL-10 production upon *Candida* stimulation was proposed as the underlying mechanism for this increased susceptibility. A similar tendency was also observed for patients who had recovered from candidaemia. These data were corroborated by the fact that IL-10 has been previously shown to inhibit the action of human monocytes against *C. albicans* (Roilides et al., 1998a). Also, in mice with disseminated candidiasis, the absence of IL-10 potentiated antifungal immunity by increasing the production of proinflammatory cytokines, leading to reduced fungal growth (Del Sero et al., 1999). The notion that TLR4 induces a more proinflammatory cytokine profile, while TLR2 has a more pronounced anti-inflammatory bias (Netea et al., 2004) may suggest that a defective TLR4-mediated signalling indirectly leads to a suppressive Th2 cytokine response through unaffected TLR2-induced IL-10 production, thus resulting in increased susceptibility to disseminated *Candida* infection.

Previous work reported no association between *TLR4* polymorphisms and vaginal colonization by *C. albicans* (Morre et al., 2002). The fact that these patients were colonized rather than having signs of active infection was suggested as underlying the lack of association. In addition, specific T cell-mediated immunity is the main line of defence against mucosal candidiasis, whereas the innate immune response, in which TLR4 recognition is crucial, is responsible for defence against disseminated candidiasis.

Although a great deal is known about the nature, ligands, interactions, genetic variants and functions of the innate immune receptors, innate immune recognition remains very complex, as it has to protect the host against a highly diverse microbial world. Their ability to discriminate among different classes of microorganisms, as well as modified host cells, remains poorly understood. Undoubtedly, the future study of these receptors will give more insights into innate immune cell biology and may provide alternative targets for the modulation of cell function for the treatment of infectious and non-infectious diseases.

CHAPTER 2

Multiplex PCR identification of eight clinically relevant *Candida* species

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"Multiplex PCR identification of eight clinically relevant *Candida* species"

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ABSTRACT

Invasive fungal infections, specifically candidaemia, constitute major public health problems with high mortality rates. Therefore, in the last few years, the development of novel diagnostic methods has been considered a critical issue. Herein we describe a multiplex PCR strategy allowing the identification of 8 clinically relevant yeasts of the *Candida* genus, namely *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *C. dubliniensis*. This method is based on the amplification of two fragments from the ITS1 and ITS2 regions by the combination of 2 yeast-specific and 8 species-specific primers in a single PCR reaction. Results from the identification of 231 clinical isolates are presented pointing to the high specificity of this procedure. Furthermore, several *Candida* isolates were identified directly from clinical specimens also attesting to the method's direct laboratory application. The results from the multiplex reactions with other microorganisms that usually co-infect patients also confirmed its high specificity in the identification of *Candida* species. Moreover, this method is simple and presents a sensitivity of approximately 2 cells per ml within 5 hours. Furthermore, it allows discrimination of individual *Candida* species within polyfungal samples. This novel method may therefore provide a clinical diagnostic procedure with direct applicability.

INTRODUCTION

Invasive fungal infections represent a public health problem of major importance (Beck-Sague and Jarvis, 1993; Fridkin and Jarvis, 1996). In particular, candidaemia has been reported within similar rates in different countries, ranging from 0.20-0.38 per 1,000 admissions and from 3.0-4.4 per 100,000 patient days (Tortorano et al., 2004). This emergence is often associated with human immunodeficiency virus (HIV) or to advanced medical and surgical interventions that compromise patient immunity, e.g., bone-marrow or solid-organ transplants, aggressive chemotherapy and broad application of antifungal agents (Fridkin and Jarvis, 1996). In fact, nosocomial fungal bloodstream infections are an increasingly significant cause of morbidity, with an estimated mortality of 25-38% (Wenzel and Edmond, 2001). *Candida albicans* is the most common and clinically relevant pathogen of the genus. However, there has been a significant trend towards the emergence of species other than *C. albicans*, with a particular increase in *C. glabrata* frequency (Diekema et al., 2002; Pfaller and Diekema, 2002; Tortorano et al., 2004) and to a lesser extent, *C. parapsilosis* and *C. tropicalis* (Kao et al., 1999). In addition, given that several non-*albicans Candida* species are intrinsically resistant to common antifungal agents, accurate identification methods are critical for the establishment of appropriate antifungal therapy (Kao et al., 1999).

The cornerstone of laboratory detection of bloodstream fungal infections, including candidaemia, remains direct examination and conventional blood culture. However, these methods are of limited clinical value since there are negative outcomes in as high as 50% autopsy-confirmed cases of candidaemia. In addition, cultures may only become positive late in the infection (Berenguer et al., 1993). Furthermore, most phenotypic methods of identification used in clinical laboratories are often time-consuming and may lead to inconclusive results. For example, phenotypic tests such as VITEK and ID32C systems require several days before biochemical reactions can be interpreted (Fenn et al., 1994). On the other hand, molecular approaches have the potential to detect candidaemia with increased sensitivity and specificity. Buchman et al. were the first to demonstrate that detection of *C. albicans* in clinical specimens was possible by PCR amplification of the lanosterol-alpha-demethylase (*L1A1*) gene (Buchman et al., 1990). Other PCR-based techniques have been developed using amplification of target DNA, providing alternative strategies for the diagnosis and identification of fungal pathogens (Burgener-Kairuz et al., 1994; Crampin and Matthews, 1993; Fujita et al., 1995; Miyakawa et al., 1992).

Fungal ribosomal genes are common DNA targets in PCR-based procedures for the identification of fungi at the species level. The highly variable sequences of internal transcribed spacer regions ITS1 and ITS2 flanked by the relatively conserved coding regions of 18S, 5.8S and 28S nuclear rRNA genes have been used in various PCR-based formats for the identification of medically important yeasts (Chang et al., 2001; Elie et al., 1998; Fujita et al., 1995; Williams et al., 1995). Even though these molecular techniques are highly sensitive and specific, their limited applicability has been the need for expensive equipment not readily accessible to many diagnostic laboratories.

We describe a rapid and simple multiplex PCR-based method able to specifically identify 8 clinically relevant *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *C. dubliniensis*) based on the amplification of particular DNA fragments of the ITS1 and ITS2 regions. The method combines two yeast-specific primers and eight *Candida* species-specific primers in a single PCR reaction yielding two amplicons of different sizes for each species. In addition, this method provides the following advantages over currently available techniques: (1) it can be used to test several clinical samples, including blood culture bottles and urine samples, (2) whole yeast cells may be employed directly in the PCR mixture, (3) it is highly specific and sensitive, with a detection limit of 2.15±0.25 cells/ml, (4) it has the potential to discriminate individual *Candida* species in polyfungal infections to a maximum ratio of 1:10, and (5) it presents good reproducibility among different PCR thermal cyclers and within different laboratories. Altogether, the features of this method point to a novel and highly advantageous application in the identification of *Candida* species in both clinical diagnosis and epidemiological studies.

MATERIALS AND METHODS

Yeast and bacterial strains. A total of 231 yeast isolates (90 *Candida albicans*, 61 *C. parapsilosis*, 25 *C. tropicalis*, 19 *C. krusei*, 18 *C. glabrata*, 13 *C. guilliermondii* and 5 *C. lusitaniae*) were recovered from clinical specimens in two Portuguese medical institutions, one in the northern region (Hospital de São João, Porto) and the other in the southern area of the country (Hospital de Santa Maria, Lisboa). The following type *Candida* strains; *C. albicans* ATCC 18804, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, *C. krusei*

ATCC 6258, *C. lusitaniae* ATCC 34449, *C. guilliermondii* ATCC 6260 and *C. dubliniensis* ATCC MYA-646 were used in the present studies. The identification of yeast isolates was carried out by conventional biochemical techniques in both medical institutions using the VITEK (bioMérieux Vitek, Inc., MO, USA) or ID32C (bioMérieux Vitek) yeast identification systems. The isolates were stored on yeast extract peptone dextrose (YEPD) agar plates. In addition, *Aspergillus fumigatus* MUM 98.02, *A. niger* MUM 03.01, *A. flavus* MUM 00.29, *A. terreus* MUM 94.09, *A. nidulans* MUM 98.32, *Cryptococcus neoformans* var. *neoformans* ATCC 28957 and *Saccharomyces cerevisiae* Y10000, as well as *Mycobacterium tuberculosis* H37Rv, *M. avium* 3509, *Escherichia coli* HB101, *Staphylococcus aureus* 0400, *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6051 were used for PCR cross-reactivity testing.

Furthermore, 27 BACTEC blood culture bottles (Becton Dickinson Microbiology Systems, MD, USA) which were identified as positive for bacteria and/or yeasts through the use of a blood culture instrument and Gram staining and 19 urine samples found to be positive for yeasts and/or bacteria were also directly analysed by PCR. Identification was simultaneously carried out using the VITEK yeast biochemical card. Twenty blood culture bottles and 14 urine samples were noted to contain yeasts, while negative controls included blood culture bottles and urine samples positive for different bacterial species. While no DNA isolation procedure was required for urine samples, *Candida* DNA was isolated from aliquots of blood culture bottles, in order to eliminate the presence of PCR inhibitory factors (see below).

Primer design. Yeast-specific universal primers UNI1 (5'-GTCAAACTTGGTCATTTA-3') and UNI2 (5'-TTCTTTTCCTCCGCTTATTGA-3') were used to amplify the internal transcribed spacer regions 1 (ITS1) and 2 (ITS2), including the 5.8S rRNA of the most relevant yeast pathogens associated with human disease (Trost et al., 2004). In addition, the species-specific primers Calb, Cgla, Ckru, Cpar, Ctro, Clus, Cgui and Cdub were designed based on the sequence data for the ITS1 and ITS2 regions of the reference strains and of all clinical isolates from the *Candida* genus available in the EMBL/GenBank databases. The sequences were compared using the DNAMAN for Windows software (Lynnon Corporation, Quebec, Canada) in order to design primers to specifically amplify DNA from *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, *C. guilliermondii* and *C. dubliniensis*, respectively (Table 1). In the multiplex PCR, the species-specific primers form a pair with universal primer UNI2, with the exception of Clus, which pairs with UNI1 (Figure 1).

Table 1. Universal and species-specific primers used in *Candida* species amplification and size of fragments visualized under agarose gel electrophoresis.

Species	Primer name	Sequence (5'-3')	Amplicon size (bp)*
Clinically relevant yeasts	UNI1	GTCAAACTTGGTCATTTA	(Trost et al., 2004)
	UNI2	TTCTTTTCCTCCGCTTATTG	
C. albicans	Calb	AGCTGCCGCCAGAGGTCTAA	583/446
C. glabrata	Cgla	TTGTCTGAGCTCGGAGAGAG	929/839
C. krusei	Ckru	CTGGCCGAGCGAACTAGACT	590/169
C. tropicalis	Ctro	GATTTGCTTAATTGCCCCAC	583/507
C. parapsilosis	Cpar	GTCAACCGATTATTTAATAG	570/370
C. guilliermondii	Cgui	TTGGCCTAGAGATAGGTTGG	668/512
C. lusitaniae	Clus	TTCGGAGCAACGCCTAACCG	433/329
C. dubliniensis	Cdub	CTCAAACCCCTAGGGTTTGG	591/217

* Amplicon sizes result from multiplex PCR amplification using yeast-specific primers (UNI1 and UNI2) and corresponding species-specific primer.

Figure 1. Multiplex PCR strategy. Organization of fungal ribosomal genes with universal and species-specific primer targets indicated. Arrows indicate the direction of PCR amplification.

DNA isolation. For DNA extraction of yeasts in culture, cells were grown overnight in YEPD medium at 26°C with aeration on a mechanical shaker (150 rpm) (Xu et al., 2000). Cells were harvested by centrifugation and the pellet suspended in 200 µl of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). For cell disruption, 200 µl of 0.5 mm-diameter glass beads and 200 µl of phenol/chloroform (1:1) were added and the tubes were shaken for three 60-second intervals interspersed with periods of cooling on ice. After disrupted cell debris was removed by a 5 min centrifugation at 3,000 *g*, the supernatant was collected and 1 ml of cold isopropanol was added before mixing by inversion. The tubes

were centrifuged for 3 min at 3,000 *g* and the sediment suspended in 400 µl of TE buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.0). A 5 min treatment with RNase A (1 mg/ml) at 37°C was then carried out before adding 10 µl of 3 M sodium acetate. DNA was further precipitated by addition of 1 ml of isopropanol, mixing by inversion and further centrifugation. Finally, the sediment was air-dried and the DNA suspended in 50 µl of ultra-filtered water. DNA content and purity were determined by spectrophotometry at 260 and 280 nm and diluted to a 100 ng/µl final concentration. The DNA isolation from whole blood and blood culture bottles was adapted from elsewhere (Flahaut et al., 1998) with minor alterations. Four hundred µl of 10× TXTE buffer (10 mM Tris-HCl, 1mM EDTA, 1% Triton X-100, pH 8.0) were added to 3.6 ml of blood sample and the mixture incubated for 20 min at room temperature to lyse blood cells. Yeast cells were collected by centrifugation at 3,000 *g* for 10 min and washed with 1× TXTE buffer and with 20 mM Tris-HCl (pH 8.3). The erythrocyte-free pellet was then suspended in 180 µl of ATL lysis buffer and 20 µl of proteinase K (1.7 mg/ml). The mixture was incubated at 65°C for 1 h, 200 µl of AL buffer were added and the sample was heated at 70°C for 10 min. After these steps, 200 µl of ethanol were added to each sample and the mixtures were applied to QIAamp mini spin columns with 2 ml collection tubes, centrifuged at 5,000 *g* for 1 min and washed twice with 500 µl of AW buffer. The columns were then washed twice with 50 mM EDTA and twice with AW buffer in order to chelate PCR inhibitory factors. DNA was eluted with 100 µl of previously heated AL buffer and kept at -20°C until PCR. ATL lysis buffer, AL buffer, AW buffer, proteinase K and the spin columns were purchased from Qiagen, Hilden, Germany.

PCR amplification. Multiplex PCR amplification was performed in a 20 µl volume consisting of 0.8× PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8)], 3.5 mM $MgCl_2$, dNTP mixture (200 µM each), primer mixture (UNI1 and UNI2, 0.55 µM each; Cgui, 0.05 µM; Calb and Ckru, 0.15 µM each; Cgla, Ctro and Clus, 0.2 µM each; Cpar, 0.3 µM; Cdub, 0.4 µM), 1 U *Taq* DNA polymerase and 50 ng genomic DNA template, with the remaining volume consisting of sterilized water. The amount of template DNA used was quantified by spectrophotometry. A 2 µl volume was used when the template was urine or isolated *Candida* DNA, either from whole blood samples or aliquots from blood culture bottles. For colony-PCR, part of a single colony was suspended directly in the PCR mixture with a sterile toothpick. PCR was routinely carried out in a Biometra Tpersonal (Whatman Biometra, Goettingen, Germany) thermal cycler under the following cycling conditions: 40 cycles of 15 s at 94°C, 30 s at 55°C,

and 45 s at 65°C, after a 10 min initial period of DNA denaturation and enzyme activation at 94°C. Additionally, multiplex PCR reactions were performed in four different thermal cyclers: iCycler and MyCycler (Bio-Rad, CA, USA), Primus 96 (MWG Biotech AG, Ebersberg, Germany) and GeneAmp PCR System 9600 (Perkin-Elmer, MA, USA) in order to confirm PCR reaction reproducibility. Negative control reactions were performed simultaneously with each test run by replacing the template DNA with sterilized water in the PCR mixture. Ten µl aliquots of each amplification product were separated by electrophoresis in a 2% agarose gel. Ethidium bromide staining (0.5 µg/ml) allowed the visualization of DNA fragments with a digital imaging system (Alpha Innotech Corporation, CA, USA) and species identification was possible by comparison with a 100-bp DNA ladder (Fermentas International Inc., Ontario, Canada). *Mycobacterium avium* subsp. *paratuberculosis* strain k10, *Mycobacterium tuberculosis* CDC1551, and *Nocardia farcinica* IFM10152 were tested using *in silico* PCR (http://www.in-silico.com/multiplex_PCR/).

Detection limit of *Candida* yeasts in whole blood. In order to determine the detection limit of *Candida* cells in whole blood by multiplex PCR, fresh human blood obtained from healthy volunteers was seeded with cells from each *Candida* species to a concentration of 5×10^2 CFU/ml. Yeast-cell number was estimated by haemocytometer counting and confirmed by plating serial dilutions of seeded blood onto Sabouraud agar plates and colony counting after 2 days of incubation at 30°C. The seeded blood was then serially diluted with whole blood to obtain yeast concentrations ranging from 2.5×10^2 to 1.25 cells/ml and 3.6 ml of the diluted samples were used for isolation of *Candida* DNA. Multiplex PCR amplification was then carried out using 2 µl of isolated DNA as template (see above).

Flow cytometry analysis. In order to eliminate the interference of DNA from dead cells, cell viability was determined for quantification of DNA in samples. Plasma membrane integrity was determined by examining cellular permeability to propidium iodide (Sigma-Aldrich Corporation, MO, USA) as described elsewhere (de la Fuente et al., 1992). Fluorescence analysis was carried out by flow cytometry with an EPICS XL-MCL (Beckman-Coulter Inc., CA, USA) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW.

RESULTS

Sequence analysis and multiplex PCR strategy. Previously designed fungus-specific universal primers UNI1 and UNI2 were used to amplify the internal transcribed spacer regions 1 (ITS1) and 2 (ITS2), including the 5.8S rRNA region (Trost et al., 2004). As shown in Figure 1 and reported by Trost and colleagues, predicted PCR products obtained by amplification using these universal primers were found to vary among *Candida* species, ranging in length from 433 bp (*C. lusitaniae*) to 929 bp (*C. glabrata*) (Trost et al., 2004). In addition, DNA from clinically relevant yeasts other than *Candida*, including species from the genus *Cryptococcus*, *Saccharomyces* and *Trichosporon* would also be amplified into a single PCR fragment using the universal primers (Trost et al., 2004). For most *Candida* species, the sizes of the obtained fragments are not sufficient to promote direct identification. Thus, species-specific variations within ITS1 and ITS2 sequences were used to design primers for amplification of an internal fragment with a lower length (Figure 1), that could allow direct identification by only a PCR reaction avoiding the restriction analysis described by Trost and collaborators (Trost et al., 2004). ITS1 and ITS2 sequences from the reference strains and from all clinical *Candida* isolates displayed in EMBL/GenBank database were analyzed by intraspecies alignments using DNAMAN for Windows software (Lynnon Corporation, Quebec, Canada) to find blocks of conserved regions among the different strains. These sequences were compared inter-species in order to find variable regions that allowed the design of specific primers to each species. Interferences from both *C. orthopsilosis* and *C. metapsilosis* in *C. parapsilosis* identification and *C. famata* and other species of the *C. guilliermondii* clade (*C. fermentati* and *C. carpophila*) in *C. guilliermondii* identification (Tavanti et al., 2005; Vaughan-Martini et al., 2005) were ruled out by sequence analysis and multiplex PCR. This *in silico* study was concluded with the design of primers in variable regions among species but at the same time conserved between strains (Table 1), thus excluding possible intraspecies variability.

Multiplex PCR amplification. In order to develop a protocol that could allow the specific identification of each *Candida* species studied in a single reaction, a simple step-by-step protocol of multiplex PCR optimization was developed (Henegariu et al., 1997). The results of multiplex PCR using isolated DNA from each species allowed the identification of up to eight clinically relevant yeasts in a single PCR reaction by the use of eight species-specific primers

together with the previously described universal primers (Trost et al., 2004) (Figure 2A). Multiplex PCR directly from living/intact yeast whole cells (commonly referred as colony-PCR (Ward, 1992)) was also assessed. PCR conditions were maintained and the DNA was replaced by whole yeast cells, where the initial 10 min at 94°C in the PCR protocol was sufficient to disrupt cell integrity allowing DNA release. Multiplex PCR reactions performed with yeast cells yielded both universal and species-specific amplicons (Figure 2B).

Identification of clinical yeast isolates. To validate the methodology described, a wide scan of 231 clinical isolates, previously characterized by conventional phenotypic systems, was carried out using the multiplex PCR-based approach. Comparison of the results of the two identification approaches (Table 2) showed discrepancies in species identification for 18 isolates representing 7.8% of the total. The multiplex PCR identification of the discrepant isolates was further confirmed using PCR fingerprinting (Figure 2E) (Correia et al., 2004), which demonstrated the accuracy of the multiplex PCR identifications. No isolates of *C. dubliniensis* were found in the clinical specimens provided by the healthcare institutions. For this reason, results for *C. dubliniensis* are not included in the agarose gel presented in Figure 2B. The most frequent misidentifications of the VITEK/ID32C analysis were of *C. parapsilosis* (7/18 of all discrepant identifications), followed by *C. albicans* (5/18), *C. tropicalis* (4/18), *C. krusei* (1/18) and *C. guilliermondii* (1/18). In addition, the most common misidentification was *C. tropicalis* for *C. parapsilosis*. Taking into account the misidentification percentage for each species regarding the number of isolates wrongly identified by the VITEK or ID32C systems, *C. tropicalis* was the most often misidentified species (16% of the *C. tropicalis* isolates), followed by *C. parapsilosis* (11.5%), *C. guilliermondii* (7.7%), *C. albicans* (5.6%) and *C. krusei* (5.3%).

Identification of *Candida* species in polymicrobial mixtures. Theoretically, the strategy here described would be able to simultaneously identify in a single multiplex PCR reaction coexisting yeast species in mixed samples. In order to experimentally assess this hypothesis, samples with two or three mixed *Candida* species were tested. The results showed that the multiplex method discriminates the different species present in polyfungal samples (Figure 2C) by the amplification of the fragments corresponding to each *Candida* species present. In fact, multiple species can be identified to a maximum cell ratio of 1:10, where for lower ratios the less abundant yeast species could not be detected (data not shown).

Table 2. Comparative results between identification in hospital centres (based on VITEK or ID32C) and identification by multiplex PCR of clinical isolates of *Candida* species. Multiplex PCR amplification of the analysed yeast species bypass the errors associated with VITEK/ID32C identification.

VITEK/ID32C identification		**Multiplex PCR identification***							**VITEK/ID32C misidentifications**
Species	Isolates	*C. albicans*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	*C. glabrata*	*C. guilliermondii*	*C. lusitaniae*	**(%)**
C. albicans	90	85	3	0	0	1	0	1	5.6 (5/90)
C. parapsilosis	61	0	54	4	0	0	1	2	11.5 (7/61)
C. tropicalis	25	0	2	21	0	2	0	0	16.0 (4/25)
C. krusei	19	0	1	0	18	0	0	0	5.3 (1/19)
C. glabrata	18	0	0	0	0	18	0	0	0 (0/18)
C. guilliermondii	13	0	1	0	0	0	12	0	7.7 (1/13)
C. lusitaniae	5	0	0	0	0	0	0	5	0 (0/5)
Total	231	85	61	25	18	21	13	8	7.8

* PCR fingerprinting (Correia et al., 2004) confirmed the results obtained by multiplex PCR.

In addition, in the case of polymicrobial samples (mixed fungal and bacterial cells), no detectable PCR products were obtained using as template *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* bacterial DNA, although *Candida* species could be identified without interference (data not shown). This confirms the specificity of the proposed methodology. In addition, common microorganisms which usually cause infection in immunocompromised populations with candidaemia, such as *Mycobacterium tuberculosis*, *M. avium* and *Nocardia farcinica* did not present cross-reactivity with *Candida* species (data not shown).

Identification of yeasts directly from urine and blood culture bottles. In order to directly apply this methodology to clinical samples, direct detection and identification of yeast cells in blood culture bottles (n=27) and urine samples (n=19) was assessed. To avoid cellular growth, the PCR reactions were performed in the samples from blood culture bottles immediately after detection of growth and in the case of urine samples, frozen aliquots were tested after positive indicators of development were obtained. The results for the *Candida* species present in the analyzed samples were in accordance to the presumptive identification provided by the healthcare institutions that carried out simultaneous phenotypic identification (Table 3). No cross-reactivity was detected in control samples positive for bacteria (7 samples) and at the same time, no interference in the identification of *Candida* species was observed in polymicrobial samples (2 samples constituted by *C. albicans* with either *S. aureus* or *P. aeruginosa*).

A step forward in the application of the multiplex PCR described would be the detection of cases of candidaemia directly from blood samples. In this sense, and to further characterize the sensibility of the methodology, the limit of detection in seeded whole blood was determined. Thus, cells of different *Candida* species were artificially inoculated in whole blood from healthy volunteers in concentrations ranging from 2.5×10^{2} to 1.25 cells/ml and DNA was isolated from a 3.6 ml sample. Cells in the exponential phase of growth were used and their viability (assessed as plasma membrane integrity) was confirmed by flow cytometry in order to exclude the presence of dead *Candida* cells that could contribute to the release of DNA, masking the sensitivity of the method (data not shown). Using the proposed multiplex protocol, the PCR products presented the characteristic mobility pattern, correctly identifying the species seeded in whole blood (Figure 2D exemplifies the case of *C. albicans*). Additionally, the sensitivity of the multiplex PCR was found to be 2.15±0.25 cells per ml of blood. On other hand, in the particular case of simulated

polyfungal infections, both species could be correctly identified to a minimum limit of approximately 20 cells/ml, although one of them was always detected to the minimum limit achieved for individual species (data not shown).

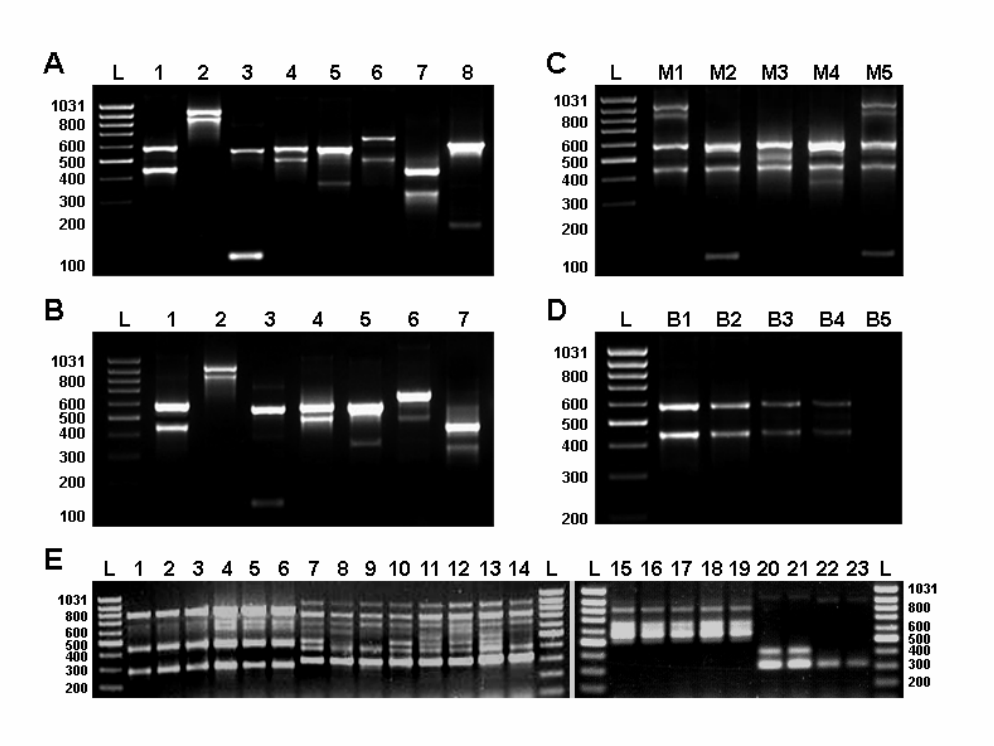

Figure 2. Agarose gel showing the results obtained for multiplex PCR of isolated yeast genomic DNA (A) or yeast whole cells (B) as template. Lanes: L – 100-bp DNA ladder; 1 - *C. albicans*, 2 - *C. glabrata*, 3 - *C. krusei*, 4 - *C. tropicalis*, 5 - *C. parapsilosis*, 6 - *C. guilliermondii*, 7 - *C. lusitaniae* and 8 - *C. dubliniensis*. (C) Multiplex PCR of *Candida* mixtures in 1:1 ratios. Lanes: L – 100-bp DNA ladder; M1 - *C. albicans* + *C. glabrata*, M2 - *C. albicans* + *C. krusei*, M3 - *C. albicans* + *C. tropicalis*, M4 - *C. albicans* + *C. parapsilosis* and M5 - *C. albicans* + *C. glabrata* + *C. krusei*. (D) Sensitivity of multiplex PCR. DNA was isolated from whole blood with different levels of artificial infection with *C. albicans*. Lanes: L – 100-bp DNA ladder; B1 – 2.5×10^{2}, B2 - 2.5×10^{1}, B3 - 5, B4 - 2.5 and B5 - 1.25 cells/ml. (E) PCR fingerprinting using primer T3B of the *Candida* isolates with different identification using multiplex PCR and VITEK/ID32C. Lanes: L – 100-bp DNA ladder; 1 – *C. albicans* type strain; 2-6 – *C. albicans* (as identified by multiplex PCR); 7 – *C. parapsilosis* type strain; 8-14 – *C. parapsilosis* (as identified by multiplex PCR); 15 - *C. tropicalis* type strain; 16-19 – *C. tropicalis* (as identified by multiplex PCR); 20 – *C. krusei* type strain; 21 – *C. krusei* (as identified by multiplex PCR); 22 – *C. guilliermondii* type strain; 23 – *C. guilliermondii* (as identified by multiplex PCR).

Table 3. Comparative results between VITEK and multiplex PCR identification of *Candida* species present in blood culture bottles and urine samples.

Clinical Samples	Total	*C. albicans*		*C. glabrata*		*C. parapsilosis*		*C. tropicalis*		Yeast-negative Identification*
		VITEK	Multiplex PCR	VITEK	Multiplex PCR	VITEK	Multiplex PCR	VITEK	Multiplex PCR	
Blood culture bottles	27	13	13	4	4	2	2	1	1	7
Urine	19	12	12	2	2	0	0	0	0	5
Total	46	25	25	6	6	2	2	1	1	12

* Yeast-negative identification was proven by both VITEK and multiplex PCR

DISCUSSION

The development of a simple approach to the identification of *Candida* species based on the combined use of universal yeast primers and *Candida* species-specific primers was undertaken. The universal primers were first reported by Trost and colleagues, who further developed a method based on the enzyme restriction of PCR fragments in order to identify a set of fungal pathogens (Trost et al., 2004). While universal primers provide a broad detection capability for yeast pathogens (Trost et al., 2004), in the present work, the presence of species-specific primers allow differentiation of *Candida* at the species level in a single PCR reaction. The multiplex PCR strategy herein described takes advantage of the high-copy number of rRNA genes, the differences in the length of ITS regions and the high variability of these regions among *Candida* species. This strategy renders the method with a test matrix able to identify eight of the most clinically relevant yeast species, including the newly emerging *C. dubliniensis*, as shown with the type strain (Figure 2A). Together, both the universal and species-specific fragments result in characteristic band patterns, enabling easy identification of the *Candida* species in question. Similarly to other genotypic-based techniques, this method allows the identification of species that have non-standard morphologic, cultural and biochemical characteristics. Additionally to the amplification of isolated DNA, the identification of living/intact whole cells in the PCR mixture was also found to be possible (Figure 2B), thus bypassing the time-consuming DNA isolation steps and reducing the time required for identification. Another advantage of this methodology resides in the rapid identification in the same multiplex PCR reaction of the aetiological agents involved in polyfungal infections (Figure 2C). Such cases involving more than one yeast have been reported to occur with a frequency of approximately 5% among all candidaemias (Pulimood et al., 2002). Furthermore, identification of the yeast species was not hampered by the presence of bacteria frequently co-infecting candidaemia patients.

The identification of *Candida* species was also carried out directly from clinical samples such as blood culture bottles and urine samples. The isolation of *Candida* DNA from aliquots of blood culture bottles required 2 h to lyse blood cells and recover DNA, 2 h for multiplex PCR amplification of isolated DNA and 1 h to perform agarose gel electrophoresis, including the time required to prepare the PCR mixture and agarose gel. Therefore, species can be identified within as little as 5 h, in contrast to phenotypic methods which can take several days (Reimer et al., 1997). Furthermore, this methodology is highly sensitive since the number of yeast cells

detectable in artificially inoculated whole blood reached a minimum limit of 2.15±0.25 cells/ml (Figure 2D). This capacity may enable the method to detect low candidaemias occurring in the early stages of infection, given that there are often less than 10 *Candida* cells circulating per ml of blood in these situations (Isenberg, 1992). Moreover, this multiplex strategy is adaptable to diverse standard equipment currently in use in most clinical laboratories and if not, likely to be easily implemented. The technique is cost-effective since it only requires essentially PCR components and DNA isolation reagents. Furthermore, no DNA probes or expensive restriction enzymes are needed. Finally, the possibility of detection of candidaemia cases directly from patients' whole blood is currently under investigation and efforts are being carried out to validate the applicability of the method in clinical settings.

CHAPTER 3

Study of disease-relevant polymorphisms in the *TLR4* and *TLR9* genes: a novel method applied to the analysis of the Portuguese population

Based on data from:

Carvalho, A., Marques, A., Maciel, P. and Rodrigues, F.
"Study of disease-relevant polymorphisms in the *TLR4* and *TLR9* genes: a novel method applied to the analysis of the Portuguese population"
Mol Cell Probes. (2007) **21**:316-20

ABSTRACT

Toll-like receptors (TLRs) are cellular receptors that mediate recognition of microbial challenges and the subsequent inflammatory response. Genetic variations within these inflammation-associated genes may alter host-pathogen defence mechanisms affecting susceptibility towards infectious diseases. Taking into account the significance of these genes, we developed a simple and rapid method based in the bi-directional PCR amplification of specific alleles (Bi-PASA) for genotyping known sequence variants in *TLR4* (Asp299Gly and Thr399Ile) and *TLR9* (T-1237C) genes. This method allows genotype determination in a single reaction and is amenable to large scale analysis. We used Bi-PASA to characterize the distribution of these polymorphisms in the Portuguese population. A total of 388 randomly selected blood donors of Portuguese origin (203 females and 185 males) were genotyped and allele frequencies were determined. Among the tested individuals, 11.1% and 10.8% were heterozygous for Asp299Gly and Thr399Ile, respectively. In what concerns the T-1237C variation in *TLR9*, the variant allele was present in 19.4% of the individuals tested. Besides confirming the usefulness of the Bi-PASA in polymorphism analysis, the data presented provide valuable information on TLR polymorphisms in the Portuguese population that can be used to stratify risk patients with increased susceptibility to infection.

INTRODUCTION

The innate immune system is able to recognize conserved motifs in pathogens in which pattern recognition receptors, including Toll-like receptors (TLRs), play an important role (Medzhitov and Janeway, 2000a). TLRs are a family of genetically conserved proteins identified as key components of the innate immune system, mediating recognition of microbial challenges and the subsequent inflammatory response (Medzhitov and Janeway, 2000b). The first of the currently known TLRs was described in 1997 as a human homologue of the *Drosophila* Toll protein, later designated TLR4 (Medzhitov et al., 1997). The complete TLR family allows the host to detect infection by most, if not all, types of microbial pathogens. Well characterized receptor-ligand pairs include TLR4 and lipopolysaccharide (LPS) (Hoshino et al., 1999; Poltorak et al., 1998), TLR5 and bacterial flagellin (Hayashi et al., 2001), TLR3 and viral double-stranded DNA (Alexopoulou et al., 2001), TLR9 and bacterial hypomethylated DNA (Hemmi et al., 2000) and TLR2, in association with TLR1 or TLR6, and a variety of cell wall components from Gram-positive bacteria (Kirschning and Schumann, 2002).

Cellular activation via TLRs triggers not only innate immune responses but also initiates adaptive immunity (Akira et al., 2001). Due to the significance of TLRs in the immune system, genetic variations within these genes could have a major impact upon host immune response to pathogens and thus, an increased susceptibility to infection. Regarding TLRs, several polymorphisms are already largely studied and characterized concerning the phenotypical outcome, such as polymorphisms in *TLR4* [A+896G (SNP ID: rs4986790) and C+1196T (SNP ID: rs4986791)]. These polymorphisms are located in the coding sequence resulting in amino acid exchanges: an aspartic acid for a glycine at position 299 (Asp299Gly) and a threonine for an isoleucine at position 399 (Thr399Ile), respectively, affecting the extracellular domain of this receptor. Others, such as those concerning *TLR9*, have just recently started to be characterized and their functional importance elucidated. Among these, the most studied is T-1237C (SNP ID: rs5743836), a polymorphism located within the putative promoter region known to influence transcriptional regulation of the *TLR9* gene.

Arbour and colleagues were the first to describe that the two mutations affecting the extracellular domain of TLR4 protein at the 299 and 399 residues were associated with blunted physiological responses to inhaled LPS (Arbour et al., 2000). A large number of association studies followed and the most relevant are summarized in Table 1.

Table 1. Summary of association studies between *TLR4/ TLR9* polymorphisms and infectious and inflammation-related diseases.

Reference	Association study	Patients (n)	Controls (n)	TLR SNP		Case vs. control (%)	p value
(Arbour et al., 2000)	Hyporesponsiveness to inhaled LPS	31	57	*TLR4*	Asp299Gly Thr399Ile	22.6 vs. 5.8	0.029
(Lorenz et al., 2002b)	Septic shock	91	73		Asp299Gly	5.5 vs. 0	0.05
(Lorenz et al., 2002a)	Premature birth associated to infection	440	351		Asp299Gly Thr399Ile	23.8 vs. 15.9	0.028
(Agnese et al., 2002)	Gram-negative infections	77	39		Asp299Gly Thr399Ile	18.0 vs. 12.8	0.004
(Tal et al., 2004)	Respiratory syncytial virus infection	99	82		Asp299Gly Thr399Ile	20.2 vs. 4.9	0.003
(Edfeldt et al., 2004)	Myocardial infarction associated to infection	1213	1561		Asp299Gly Thr399Ile	10.7 vs. 7.9	0.004
(Hawn et al., 2005)	Legionnaire's disease	108	510		Asp299Gly Thr399Ile	2.5 vs. 6.5	0.025
(Rezazadeh et al., 2006)	Brucellosis	198	111		Asp299Gly	33.6 vs. 20.7	<0.0001
(Mockenhaupt et al., 2006)	Severe malaria	70	51		Thr399Ile	24.1 vs. 17.6	<0.05
(Lazarus et al., 2003)	Asthma	67	152	*TLR9*	T-1237C	23.4 vs. 14.5	0.042
(Torok et al., 2004)	Crohn's disease	174	265		T-1237C	19.3 vs. 11.9	0.0036

Since their identification, the Asp299Gly and Thr399Ile mutations have been studied for their association with various infectious and inflammatory diseases. Specifically, a positive association of the Asp299Gly mutation with increased susceptibility to Gram-negative bacteraemia and septic shock was found (Agnese et al., 2002; Lorenz et al., 2002b). Interestingly however, no association between the Asp299Gly and/or Thr399Ile mutations was found for other diseases of infectious aetiology, including candidiasis (Morre et al., 2002), tuberculosis (Newport et al., 2004) or meningococcal disease (Read et al., 2001). The variation T-1237C in the promoter region of *TLR9*, on the other hand, has been shown to be associated with an increased risk for asthma (Lazarus et al., 2003) and preliminary data regarding a possible positive association with Crohn's disease was also reported (Torok et al., 2004). Nevertheless, some studies also reported no association between this polymorphism and susceptibility to systemic lupus erythematosus (De Jager et al., 2006) and atopy (Berghofer et al., 2005).

Thus, assessment of polymorphisms in TLRs may have a potential clinically usefulness for risk stratification of patients possibly more vulnerable to infections. Some of the previously developed methods are, to a certain extent, attractive laboratory-tailored approaches for genotyping polymorphisms in TLR genes, such as the real-time PCR-based method developed by Hamann et al. (Hamann et al., 2004) or the single tube PCR reaction based on exonuclease degradation of allele-specific probes described by van Rijn et al. (van Rijn et al., 2004). However, most of the genotyping techniques currently available are still either time-consuming and laborious or require expensive equipment (Heesen et al., 2003; Lorenz et al., 2001a; Schmitt et al., 2002; van Rijn et al., 2004). In this sense, we adapted a simple and rapid method, allowing genotype determination in a single reaction based in the bi-directional PCR amplification of specific alleles (Bi-PASA) (Liu et al., 1997) in both the *TLR4* (Asp299Gly, Thr399Ile) and *TLR9* (T-1237C) genes, a methodology amenable to large scale analysis, using technology available in most scientific laboratories.

MATERIALS AND METHODS

Bi-PASA uses a combination of four primers, two outer primers (named P and Q) and two inner allele-specific primers (termed M and W). The inner primers are characterized by containing

a 10-nucleotide G+C-rich 5' tail, which prevents "megapriming" and enhances the efficiency of amplification. Depending on the genotype, Bi-PASA produces two or three overlapping fragments. PQ is always produced and serves as a positive control. PW and MQ are present in a heterozygote individual, but PW is only produced in wild-type homozygote and MQ only in homozygous mutant samples (Figure 1).

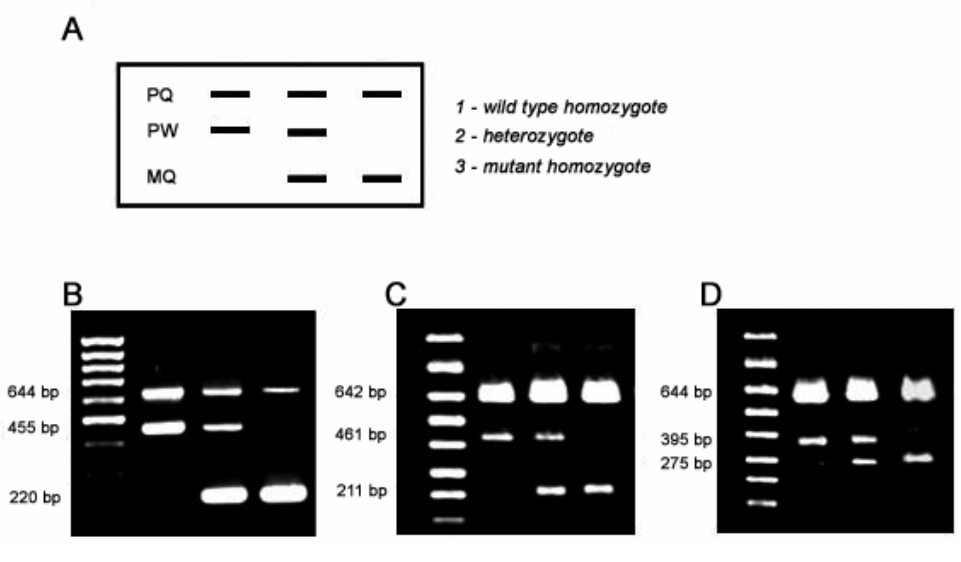

Figure 1. Predicted band pattern obtained with Bi-PASA genotyping (A). Agarose gel electrophoresis showing results of Bi-PASA genotyping for Asp299Gly (B), Thr399Ile (C) and T-1237C (D).

Primers for Bi-PASA were designed following the guidelines proposed by Liu and colleagues (Liu et al., 1997), taking into account both the melting temperature of both the primers and the largest PCR segment (PQ). The primers used in this study are shown in Table 2. The Bi-PASA methodology was optimized using DNA samples from individuals with known genotype for the polymorphisms being studied (Figure 1B-D). These samples were previously genotyped by Berghöfer et al. and Hamann et al. using real-time PCR-based methodologies and confirmed by direct sequencing (Berghofer et al., 2005; Hamann et al., 2004).

DNA was isolated from whole blood samples using the salting-out procedure described by Miller and colleagues (Miller et al., 1988) and PCR amplification was performed in a 20 µl volume that included autoclaved ultra-filtered water, PCR buffer (1.6×), dNTP mixture (200 µM each), primers (0.05-0.4 µM each; for details see Table 2), *Taq* DNA polymerase (1 U/20 µl) and approximately 50 ng genomic DNA templates. PCR cycling conditions included 35 cycles of 15 s

at 94°C, 30 s at 57°C (or 48°C for Asp299Gly), and 45 s at 65°C, after a 10-min initial period of DNA denaturation and enzyme activation at 94°C. The amplified fragments had sizes readily distinguishable by electrophoresis through a 2% agarose gel. The results obtained for the genotype of the Portuguese population by Bi-PASA were further validated by sequencing analysis of 10 randomly selected samples (data not shown).

Table 2. Bi-PASA primers used in this study.

Gene	Polymorphism	Primer	Primer final concentration (μM)
TLR4	Asp299Gly	P: 5'-AGAACTTAATGTGGCTCACAAT-3'	0.1
		Q: 5'-GAAAAAGCATTCCCACCTTTG-3'	0.1
		W: 5'- ggcggcggggTTAAATAAGTCAATAATAT-3'	0.4
		M: 5'- gggccgggggTACTACCTCGATGG-3'	0.4
	Thr399Ile	P: 5'-CTGGCTGGTTTAGAAGTCCA-3'	0.1
		Q: 5'-ATTGAAAGCAACTCTGGTGTG-3'	0.1
		W: 5'-ggcggcggggAAATACTTTAGGCTG-3'	0.1
		M: 5'-gggccgggggTGATTTTGGGACAAT-3'	0.1
TLR9	T-1237C	P: 5'-TCATTCAGCCTTCACTCAGA-3'	0.4
		Q: 5'-CACATTCAGCCCCTAGAGGG-3'	0.4
		W: 5'-ggcggcggggGTGCTGTTCCCTCTGCCTGA-3'	0.05
		M: 5'-gggccgggggATGAGACTTGGGGGAGTTTC-3'	0.05

RESULTS AND DISCUSSION

After written informed consent was obtained from each volunteer, a total of 388 randomly selected blood donors of Portuguese origin (203 females and 185 males) were genotyped using Bi-PASA. Among the tested individuals, 43/388 (11.1%) and 42/388 (10.8%) were heterozygous for Asp299Gly and Thr399Ile polymorphisms, respectively. None of the individuals showed a homozygous *TLR4* polymorphism. In what concerns the T-1237C variation in *TLR9*, 67/388 (17.3%) were heterozygous and 8/388 (2.1%) were homozygous for this polymorphism. Based on the control samples of a large number of published studies (Vogel et

al., 2005), the two *TLR4* polymorphisms, Asp299Gly and Thr399Ile, are known to be present at an overall allele frequency of 6.4±2.8 (range, 0-19.6; n=38 studies) and 7.2±3.8 (range, 0-13, n=11), respectively. However, reports of differences in the frequencies of these two polymorphisms among different ethnic groups have been published (Newport et al., 2004; Smirnova et al., 2001). In fact, considering the data currently available on the Entrez SNP database (Table 3), the frequency of *TLR4* Asp299Gly polymorphism is much higher in African Americans than in Europeans and sub-Saharan Africans, in contrast to Asians, where this particular polymorphism does not seem to occur. The overall frequencies of Thr399Ile are similar to that of Asp299Gly, although no data is available for African Americans. African Americans also display a higher frequency of the *TLR9* polymorphism than Europeans and Asians. Comparing with our results, we can observe that *TLR4* Asp299Gly and Thr399Ile allele frequencies in the Portuguese population are higher than those described for the European population characterized in the Entrez database, although similar to those of controls published in association studies using European populations. Concerning *TLR9*, the frequency of T-1237C in the Portuguese population is similar to the one presented in the database for the European population.

Table 3. Allele frequencies of each TLR polymorphism in distinct ethnic populations [data from Entrez SNP database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp) in November, 2006] and in our study in the Portuguese population.

TLR SNP		**Allele frequencies**				
		This study	European	Asian	African American	Sub-Saharan African
Asp299Gly	A	**0.889**	0.967	1.000	0.848	0.967
	G	**0.111**	0.033	0	0.152	0.033
Thr399Ile	C	**0.892**	0.967	1.000	n/a	1.000
	T	**0.108**	0.033	0		0
T-1237C	C	**0.806**	0.841	0.974	0.750	n/a
	T	**0.194**	0.159	0.026	0.250	

n/a – not available

Distributions of both *TLR4* and *TLR9* genotypes did not deviate from those predicted by the Hardy-Weinberg equilibrium (Asp299Gly, p=0.388; Thr399Ile, p=0.410; T-1237C, p=0.58). In addition and as expected, both *TLR4* polymorphisms are in total linkage disequilibrium (D'=1.000). In fact, co-segregation among these two mutations has also been largely stated (Hartel et al., 2004; Lorenz et al., 2002b; Tal et al., 2004).

In conclusion, the data herein presented provides valuable information on TLR polymorphisms in the Portuguese population that can be used in future studies to stratify patients regarding susceptibility to infection. Our results clearly show that Bi-PASA is a valuable methodology for genotyping studies. In addition, this method can easily be applied in studies of large populations, and further applied for the study of other polymorphisms, as it is a cost-effective technique, where in a single reaction the genotype of the individuals can be determined.

CHAPTER 4

Polymorphisms in Toll-like receptor genes and susceptibility to pulmonary aspergillosis

Based on data from:

Carvalho, A., Pasqualotto, A.C., Pitzurra, L., Romani, L., Denning, D.W. and Rodrigues, F. "Polymorphisms in Toll-like receptor genes and susceptibility to pulmonary aspergillosis" *J Infect Dis.* (2008) **197**:618-21

ABSTRACT

Toll-like receptors (TLRs) are important components of innate immunity. While certain genetic variations have been associated with invasive aspergillosis, none are described for chronic pulmonary or allergic aspergillosis. We investigated the association between polymorphisms in the *TLR2*, *TLR4*, and *TLR9* genes and susceptibility to non-invasive forms of pulmonary aspergillosis. A significant association was observed between allele G on Asp299Gly (*TLR4*) and chronic cavitary pulmonary aspergillosis (p=0.003; odds ratio [OR], 3.46). In addition, susceptibility to allergic bronchopulmonary aspergillosis was associated with allele C on T-1237C (*TLR9*) (p=0.043; OR, 2.49). No particular polymorphism was associated with severe asthma with fungal sensitization. These findings differ from those described in invasive aspergillosis, suggesting important innate immune differences in the pathogenesis of different forms of aspergillosis.

INTRODUCTION

Aspergillus fumigatus is the most prevalent airborne, filamentous fungal pathogen in humans. It is the main cause of aspergillosis, an important condition that can be manifested as invasive, chronic non-invasive and allergic syndromes (Denning, 1998). Allergic bronchopulmonary aspergillosis (ABPA) is an allergic hypersensitivity to bronchial colonization by *Aspergillus* species, which affects mainly patients with asthma and patients with cystic fibrosis (Greenberger, 2003). A severe asthma phenotype associated with fungal sensitization (SAFS), distinguished from ABPA by lower IgE levels, can also develop. In contrast, chronic cavitary pulmonary aspergillosis (CCPA) is a subacute and slowly destructive infection form of pulmonary aspergillosis.

Although the immunological status of the host is probably the main determinant of the clinical presentation of aspergillosis, very little is known about this subject. Protective defence against pathogenic fungi requires effective innate and adaptive (antigen-specific) immune responses. In recent years, considerable attention has focused on the importance of innate immunity, the first line of defence which differentiates self from non-self and activates the adaptive immune system through specific signals (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997). Genetic studies revealing additional risk factors for aspergillosis are needed, mainly for patients with no obvious predisposing conditions such as neutropenia and receipt of corticosteroids. The latest evidence has focused on proteins that belong to the collectin family, including the lung surfactant protein (SP), mannose-binding lectin (MBL), and pentraxin 3 (PTX3). Allele variants in a gene encoding SP-A have been associated with increased susceptibility to both ABPA and CCPA (Saxena et al., 2003; Vaid et al., 2007). In addition, treatment with SP-A or SP-D was shown to be protective against pulmonary hypersensitivity induced by *A. fumigatus*, by lowering blood eosinophilia, pulmonary infiltration and specific antibody levels (Madan et al., 2001). Also, distinct MBL genotypes have been linked with CCPA (Crosdale et al., 2001; Vaid et al., 2007), and an intronic polymorphism in the *MBL* gene was found to underlie elevated levels of MBL and greater disease severity in ABPA patients (Kaur et al., 2006). On the other hand, PTX3 was shown to play a non-redundant role in resistance to *Aspergillus*, with PTX3-null mice showing increased susceptibility to invasive pulmonary aspergillosis (Garlanda et al., 2002). In addition, immunocompromised mice with invasive aspergillosis have been successfully treated

with PTX3 (Gaziano et al., 2004). In conjunction, these exciting data revealed the importance of innate immunity in the host response against *Aspergillus* infections.

The host innate immune system is able to recognize conserved motifs in fungal pathogens through pattern recognition receptors, including Toll-like receptors (TLRs) (Medzhitov and Janeway, 1998). TLRs are transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats (LRR) and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, which activates common signalling pathways. TLRs mediate the recognition of microbial challenges and the subsequent inflammatory response through rapid changes in the expression of genes encoding cytokines and inflammatory molecules (Akira et al., 2001). More specifically, TLR2 and TLR4 have been implicated as important components of the initial host immune response to fungal pathogens, both yeasts and moulds (Romani, 2004).

Several variations in TLR genes have been studied, including that of Asp299Gly (A+896G) in the *TLR4* gene (Schroder and Schumann, 2005). This results in an amino acid change in the LRR domain of TLR4, impairing its recognition ability. This single nucleotide polymorphism (SNP) has been correlated with diminished airway response to inhaled lipopolysaccharide in healthy individuals (Arbour et al., 2000). On the other hand, the Arg753Gln (G+2258A) polymorphism, which affects the TIR domain of TLR2, impairs signal transduction and its functional activity. Another SNP, T-1237C, located within the putative promoter region of the *TLR9* gene has been implicated in chronic inflammatory diseases including asthma (Lazarus et al., 2003) and Crohn's disease (Torok et al., 2004). Moreover, TLRs have also been associated with several pathological conditions affecting the lungs (Basu and Fenton, 2004). Thus, due to the significance of innate immunity in host defence, genetic variations in the genes of this system could have a major impact on immune responses to *Aspergillus* infections.

In the present population-based, case-control study, we aimed to explore the contribution made by polymorphisms in the *TLR2*, *TLR4* and *TLR9* genes to susceptibility to different forms of aspergillosis, namely CCPA, ABPA, and SAFS.

MATERIALS AND METHODS

Study population. The study population comprised 76 patients; males and females were equally distributed. Mean age (±SD) was 57.6 (±11.8) years. The main diagnoses were

CCPA (n=40), ABPA (n=22), and SAFS (n=14). These individuals were recruited from a cohort of patients attending South Manchester University Hospitals NHS Trust (SMUHT, UK) for *Aspergillus*-related diseases. Eighty unrelated healthy individuals of identical ethnicity were included as controls. These controls gave no previous history of pulmonary disease. Informed written consent was obtained from all participants.

Clinical evaluation. The clinical definitions were as follows (Denning et al., 2003). CCPA was diagnosed in the presence of the following symptoms and/or results: (1) chronic pulmonary or systemic symptoms with exclusion of other pulmonary pathogens, (2) radiological evidence of progressive pulmonary lesions with surrounding inflammation (with or without an intracavitary mass), (3) precipitating (IgG) antibody to *Aspergillus* in the serum, and (4) persistently elevated inflammatory markers (e.g., C-reactive protein, erythrocyte sedimentation rate, or plasma viscosity).

The diagnostic criteria for ABPA included the following symptoms and/or results (Tillie-Leblond and Tonnel, 2005): (1) asthma, (2) total serum IgE concentration ≥1000 IU/ml, (3) elevated *A. fumigatus*-specific serum IgE levels, (4) precipitating antibodies to *A. fumigatus* in the serum (not always present in patients with long-standing ABPA), and (5) central bronchiectasis. The minor diagnostic criteria for ABPA were as follows: peripheral blood eosinophilia (often absent in patients on steroids), repeated detection of *Aspergillus* in sputum, expectoration of brown plugs or flecks, and history of recurrent pulmonary infiltrates (transient or fixed).

The diagnosis of SAFS was based on the basis of the following recently proposed criteria (Denning et al., 2006): (1) severe asthma, (2) total IgE <1,000 IU/ml and (3) positive skin prick test and/or raised specific IgE to *A. fumigatus*. We included in this study only SAFS who reacted to *A. fumigatus*. In contrast to patients with ABPA, patients with SAFS usually do not give a history of productive cough containing mucous plugs and infrequently have positive sputum cultures for fungi.

Genotyping of *TLR2*, *TLR4* and *TLR9* polymorphisms. Genotype determination of polymorphisms in *TLR2* (Arg753Gln; SNP id: rs5743708), *TLR4* (Asp299Gly; SNP id: rs4986790) and *TLR9* (T-1237C; SNP id: rs5743836) genes was performed by use of bi-directional PCR amplification of specific alleles (Bi-PASA), as described elsewhere (Carvalho et al., 2007). Details about the primers used in this study are shown in Table 1.

Table 1. PCR primers used to analyse polymorphisms in *TLR2*, *TLR4* and *TLR9* genes by use of Bi-PASA genotyping.

Target gene, SNP	Primer sequence (5'→3')	Primer concentration, μM	DNA fragment sizes, bp
TLR2 Arg753Gln	P: CTCCAGGCCAAAAGGAAGC	0.1	PQ: 524 PW: 399 MQ: 152
	Q: AAAGATCCCAACTAGACAAAGA	0.1	
	W: ggcggcgggggccTGTTCATTATCTTCC	0.1	
	M: gggccgggggTTCTGCAAGCTGCA	0.1	
TLR4 Asp299Gly	P: AGAACTTAATGTGGCTCACAAT	0.1	PQ: 644 PW: 455 MQ: 220
	Q: GAAAAAGCATTCCCACCTTTG	0.1	
	W: ggcggcgggggccTTAAATAAGTCAATAATAT	0.4	
	M: gggccgggggTACTACCTCGATGG	0.4	
TLR9 T-1237C	P: TCATTCAGCCTTCACTCAGA	0.4	PQ: 644 PW: 395 MQ: 275
	Q: CACATTCAGCCCCTAGAGGG	0.4	
	W: ggcggcgggggccTGCTGTTCCCTCTGCCTGA	0.05	
	M: gggccgggggATGAGACTTGGGGGAGTTTC	0.05	

NOTE. P, left outer primer; Q, right outer primer; W, inner wild-type primer; M, inner mutant primer

Statistical analysis. The Fisher exact test and Pearson χ^2 test were used to compare allele frequencies between patient groups and controls. Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using a χ^2 test on a contingency table of observed *vs.* predicted genotype frequencies. χ^2 values, odds ratios, and p values were calculated with 95% confidence intervals.

RESULTS

TLR polymorphisms in the control population were characterized as follows (Table 2). Of the 80 control patients studied, 5 of 80 (6.2%), 10 of 80 (12.5%), and 15 of 80 (18.8%) patients were heterozygous for Arg753Gln (G/A), Asp299Gly (A/G), and T-1237C (T/C) polymorphisms, respectively. In addition, no homozygous mutation was observed in controls (i.e., A/A for

Arg753Gln, G/G for Asp299Gly, or C/C for T-1237C). The distribution of genotypes did not deviate from those predicted by the Hardy-Weinberg equilibrium.

There was no significant difference in patients with ABPA and control patients with respect to allele frequencies or genotype distribution of the *TLR2* and *TLR4* polymorphisms (Table 2). The G/A genotype in *TLR2* was not detected in any patient with ABPA. Although patients with ABPA had a lower frequency of the G allele in *TLR4*, compared with control patients (2.3% vs. 6.3%), this difference did not reach statistical significance (p=0.463). Patients with ABPA had a significantly higher frequency of allele C for the T-1237C SNP in *TLR9* than control patients (20.5% vs. 9.4%; p=0.043; odds ratio [OR], 2.49).

The frequency of allele A for the *TLR2* polymorphism among patients with SAFS was more than twice that observed among control patients (7.1% vs. 3.1%), although this difference was not statistically significant (p=0.280). No difference was observed regarding the *TLR4* variation between patients with SAFS and control patients; both presented similar allele frequencies. Patients with SAFS had a lower frequency of allele C in the *TLR9* gene, although this difference was nonsignificant in comparison with control patients (3.6% vs. 9.4%; p=0.474). When patients with SAFS and ABPA were considered together and compared to control patients, no difference in allele frequency in the TLR genes was observed (data not shown).

Patients with CCPA demonstrated a statistically significant higher frequency of allele G in the *TLR4* gene, compared with controls (18.8% vs. 6.3%; p=0.003; OR, 3.46). The frequency of SNPs in the *TLR2* and *TLR9* genes was similar for patients with CCPA and control patients.

Table 2. Distribution of genotypes and allele frequencies of the studied polymorphisms in *TLR2*, *TLR4*, *TLR9* genes in study and control patients.

Gene, SNP	Study group (*n*)	Genotype *n* (% frequency)			Allele *n* (% frequency)		χ^2	p	OR (95% CI)
		G/G	**G/A**	**A/A**	**G**	**A**			
TLR2 Arg753Gln	Controls (80)	75 (93.8)	5 (6.2)	0 (0.0)	155 (96.9)	5 (3.1)			
	ABPA (22)	22 (100)	0 (0.0)	0 (0.0)	44 (100.0)	0 (0.0)	1.41	0.587	1.032 (1.004-1.061)
	SAFS (14)	12 (85.7)	2 (14.3)	0 (0.0)	26 (92.9)	2 (7.1)	1.07	0.280	2.385 (0.439-12.944)
	CCPA (40)	38 (95.0)	2 (5.0)	0 (0.0)	78 (97.5)	2 (2.5)	0.07	1.000	0.795 (0.151-4.190)
		A/A	**A/G**	**G/G**	**A**	**G**			
TLR4 Asp299Gly	Controls (80)	70 (87.5)	10 (12.5)	0 (0.0)	150 (93.8)	10 (6.3)			
	ABPA (22)	21 (95.5)	1 (4.5)	0 (0.0)	43 (97.7)	1 (2.3)	1.07	0.463	0.349 (0.043-2.802)
	SAFS (14)	12 (85.7)	2 (14.3)	0 (0.0)	26 (92.9)	2 (7.1)	0.03	0.695	1.154 (0.239-5.570)
	CCPA (40)	25 (62.5)	**15 (37.5)**	0 (0.0)	65 (81.3)	**15 (18.8)**	8.93	0.003	3.462 (1.477-8.110)
		T/T	**T/C**	**C/C**	**T**	**C**			
TLR9 T-1237C	Controls (80)	65 (81.2)	15 (18.8)	0 (0.0)	145 (90.6)	15 (9.4)			
	ABPA (22)	14 (63.6)	7 (31.8)	1 (4.54)	35 (79.5)	**9 (20.5)**	4.08	0.043	2.486 (1.005-6.145)
	SAFS (14)	13 (92.9)	1 (7.1)	0 (0.0)	27 (96.4)	1 (3.6)	0.03	0.474	0.358 (0.045-2.825)
	CCPA (40)	33 (82.5)	7 (17.5)	0 (0.0)	73 (91.3)	7 (8.8)	0.02	0.874	0.927 (0.362-2.373)

NOTE. ABPA, allergic bronchopulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; CI, confidence interval; OR, odds ratio, SAFS, severe asthma with fungal sensitization. χ^2 and *P* values were calculated regarding allele frequencies.

DISCUSSION

TLR4 is among the major receptors involved in the recognition of pathogenic fungi and the initiation of the inflammatory response (Bellocchio et al., 2004b; Braedel et al., 2004; Tillie-Leblond and Tonnel, 2005; Wang et al., 2001b). Wang et al. proposed TLR4 as the main receptor for *Aspergillus* hyphae, because monoclonal antibodies directed against CD14 and TLR4 partially inhibited TNF-α release from human monocytes stimulated by *A. fumigatus* hyphae (Wang et al., 2001b). Accordingly, TLR4-deficient mice have shown increased susceptibility to *A. fumigatus* infection (Netea et al., 2003). Netea et al. showed that macrophages from TLR4-deficient mice produced less TNF-α and IL-1 than did macrophages from control mice upon stimulation with *A. fumigatus* conidia but not with hyphae (Netea et al., 2003). These data suggest that TLR4-mediated signals might be lost during *Aspergillus* germination, therefore implying that this phenotypic switching might be a mechanism to escape the immune system. Interestingly, we found the Asp299Gly polymorphism (*TLR4*) to be highly associated with CCPA ($p<0.01$; OR, 3.46). This SNP has not been previously observed to be associated with acute invasive aspergillosis (Kesh et al., 2005). Because its presence does not ultimately affect signal transduction and cytokine production in mononuclear cells challenged with *A. fumigatus* (van der Graaf et al., 2005), a putative mechanism for disease association would be an abnormal TLR4 extracellular domain, which hampered its function by disrupting microbial recognition. Patients with CCPA appear to have multiple defective immune responses, of which this may be one in a minority of patients. On the other hand, allele G on Asp299Gly was slightly less frequent in ABPA patients. This is perhaps unexpected, as it has been shown to be protective in hyper-inflammatory states, such as atherosclerosis, by diminishing the levels of proinflammatory cytokines (Kiechl et al., 2002). The significance, if any, of this observation requires a study involving a larger number of patients with ABPA for clarification.

We observed no association between *TLR2* polymorphisms and susceptibility to CCPA. Similar findings were previously described for patients with invasive aspergillosis (Kesh et al., 2005). Interestingly, the *TLR2* SNP was not present in any patient with ABPA, which could argue for a protective role against ABPA. It is curious in this regard that TLR2-deficient mice are highly susceptible to experimental ABPA (L. Romani, unpublished observations). Ultimately, the numbers in our study population were too small to show any significant difference, suggesting at

maximum a weak effect. For the same reason, one can speculate that allele A might increase the risk for SAFS.

Also interesting was the association of ABPA with the *TLR9* SNP ($p<0.05$; OR, 2.49). TLR9 is a receptor that detects unmethylated CpG motifs prevalent in bacterial and viral DNA (Hemmi et al., 2000). As *A. nidulans* has been shown to be virtually devoid of genomic cytosine methylation (Antequera et al., 1984) (which is probably also true for *A. fumigatus*), TLR9 activation by CpG motifs may occur after lysis of fungal cells during *Aspergillus* infections. However, the importance of TLR9 in aspergillosis is still obscure. Although both conidia and hyphae of *A. fumigatus* seem to signal through TLR9 on murine neutrophils (Bellocchio et al., 2004b), TLR9-deficient mice showed paradoxically greater conidiocidal activity and hyphal damage than wild-type mice. In addition, TLR9 has been associated with anti-allergic activities (Racila and Kline, 2005). Accordingly, the *TLR9* SNP has been associated with an increased risk of asthma (Lazarus et al., 2003), and TLR9 agonists are under development for patients with asthma and other allergic conditions. It remains to be elucidated if this constitutes an independent risk factor for ABPA, since all ABPA patients in our cohort were also diagnosed with asthma. In addition, work by Novak et al. (Novak et al., 2007) showed that the C allele of T-1237C decreases *TLR9* expression, which could also be proposed as a feature underlying susceptibility to ABPA.

Due to the limited number of patients in the study, the importance of the *TLR2* SNP in patients with aspergillosis cannot be underestimated, although its association with CCPA seems statistically improbable. No polymorphism was associated with the SAFS phenotype, reflecting either the limited number of patients, or more likely different pathogenesis. Also, the importance of polymorphisms affecting other TLR genes (as well as other SNPs in the TLRs investigated here) cannot be excluded. Because multiple receptors appear to be involved in the recognition of different components of *Aspergillus*, a better understanding of the signalling processes involved in innate immunity is ultimately required.

CHAPTER 5

Polymorphisms in Toll-like receptor genes and susceptibility to infections in allogeneic stem cell transplantation

Based on data from:

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ABSTRACT

The discovery of genetic variations in the genes encoding for Toll-like receptors (TLRs) has highlighted a potential link between genomic variation of the host and susceptibility to infections. We investigated the association between polymorphisms in the *TLR2*, *TLR4* and *TLR9* genes in recipients of allogeneic haematopoietic stem cell transplant and susceptibility to infections caused by cytomegalovirus and filamentous fungi. A significant association was observed between the presence of allele C on the T-1237C polymorphism (*TLR9*) and susceptibility to viral pneumonia (p=0.02; OR, 2.83). For fungi, a significant association was observed between the presence of allele G on the Asp299Gly polymorphism (*TLR4*) and fungal colonization (p=0.02; OR, 7.5). However, susceptibility to fungal infections, predominantly fungal pneumonia, was instead significantly decreased in the presence of allele G on the Asp299Gly polymorphism (p=0.01; OR, 0.19). Thus, fungal colonization may not predict susceptibility to infection in the presence of this SNP. The finding that defective viral but not fungal sensing may predict susceptibility to infections highlights the divergent function of TLRs in the pathogenesis of opportunistic infections.

INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) is an important option in the management of many haematological disorders (Welniak et al., 2007). However, although significant advances have been made in donor-recipient matching, conditioning regimens and post-transplant immunosuppressive therapy, HSCT is associated with a risk of complications that include graft-versus-host disease (GvHD) and viral (cytomegalovirus) or fungal (mainly *Aspergillus* spp.) infection (Cappellano et al., 2007; Pagano et al., 2007). The finding that only a fraction of patients with similar degrees of immunosuppression develop infection suggests that other, yet to be defined factors, contribute to susceptibility to infections. Recent studies have shown the association between polymorphic features of non-HLA cytokine encoding genes and the incidence and severity of post-transplant complications in the recipients of allogeneic HSCT, implying that the donor-recipient genotyping, extended for immune response loci, may be of prognostic value for the transplantation outcome (Bogunia-Kubik et al., 2006; Chen et al., 2006; Mayor et al., 2007).

There is growing evidence that variations within the genes of the family of innate immune receptors may account in part for the inherited differences in infectious disease susceptibility (Schroder and Schumann, 2005; Schwartz and Cook, 2005). The Toll-like receptor (TLR) family regulates both innate and adaptive immune responses (Akira et al., 2006). Given its broad effect on immunity, the function of TLRs in various human diseases has been investigated largely by comparing the incidence of disease among individuals with different polymorphisms in the genes that participate in TLR signalling. Growing amounts of data suggest that the ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphisms (SNPs) within TLR genes, resulting in an altered susceptibility to, or course of, infectious or inflammatory disease (Lasker and Nair, 2006; Lazarus et al., 2002). Two polymorphisms in the coding region of *TLR2*, which attenuate receptor signalling, enhance the risk of acute severe infections, tuberculosis, and leprosy (Texereau et al., 2005). For TLR9, although it is highly conserved across species, differences in optimal stimulatory motifs imply that genetic variation could play a role in human diseases associated with altered innate immune responses (Krieg, 2006; Vollmer, 2006). The *TLR9* SNP, T-1237C, located within the putative promoter region of the *TLR9* gene, has been implicated in chronic inflammatory diseases such as asthma (Lazarus et al., 2003) and Crohn's disease (Torok et al., 2004).

Most studies, however, have focused on the highly polymorphic *TLR4* gene which encodes the receptor recognizing bacterial lipopolysaccharide (LPS). Two cosegregating missense polymorphisms affecting the extracellular domain have been identified in the *TLR4* gene at minor allele frequencies between 8 and 10% in Caucasian populations (Lorenz et al., 2001b), which result, respectively, in aspartic acid to glycine substitution at position 299 (Asp299Gly) and threonine to isoleucine substitution at position 399 (Thr399Ile) in the receptor protein. These polymorphisms have been linked with blunted airway (Arbour et al., 2000) and systemic inflammatory responses (Michel et al., 2003) to inhaled LPS in adults and attenuated LPS-induced responses in primary airway epithelial cells (Arbour et al., 2000). However, these SNPs seem to protect from atherosclerosis and related diseases (Li and Sun, 2007) and, regarding the Asp299Gly SNP, from malaria (Ferwerda et al., 2007).

While reinforcing the role of TLRs in immunity and host resistance to fungi (Romani, 2004), clinical evidence suggest that SNPs in selected TLR genes are associated with susceptibility to fungal infections (Morre et al., 2002; Van der Graaf et al., 2006), including in HSCT, where recipient but not donor SNPs were associated with susceptibility to invasive aspergillosis (Kesh et al., 2005). Given the role of innate immunity in the overall immune reconstitution after HSCT (Chen et al., 2006; Land, 2007), including rebuilding of antimicrobial immunity, genetic variations in innate immunity genes could have a major impact on microbial detection and ensuing immune response.

In the present case-control study, we investigated the contribution made by selected polymorphisms in *TLR2*, *TLR4* and *TLR9* genes to susceptibility to fungal or viral infection in patients undergoing HSCT.

MATERIALS AND METHODS

Study population. A total of 65 patients were included in the study, with males and females equally distributed. Mean age (±SD) was 41.0 (±16.1) years. Diagnoses of haematological disease included aplastic anaemia (n=1), acute lymphoblastic leukaemia (n=10), acute myeloid leukaemia (n=33), chronic lymphoid leukaemia (n=2), Hodgkin lymphoma (n=8), non-Hodgkin lymphoma (n=6), and multiple myeloma (n=5). Patients were recruited from a cohort of patients attending the Haematopoietic Stem Cell Transplant Unit, Section of

Haematology and Immunology, Department of Clinical and Experimental Medicine, University of Perugia, Italy. After signing informed consent, DNA was extracted as described (Carvalho et al., 2007) from 5-10 ml of peripheral blood from patients after full chimerism was documented (a month after stem cell infusion) (Aversa et al., 1998). Donors were HLA-identical siblings in 28 patients and one-haplotype mismatched family members in the others. Graft processing and transplantation procedure was previously described (Aversa et al., 1998; Aversa et al., 2005; Tabilio et al., 1997).

Microbiological evaluation. Patients were monitored for fungal colonization twice weekly, after transplant. Positive fungal cultures (39 patients) referred to positive tests on specimens from blood, bronchial alveolar lavage, sputum, stool, urine and catheter tips and no report of negative results for repeated tests on specimen from the same site. *Aspergillus fumigatus* (n=31), *Aspergillus niger* (n=3), *Penicillium* spp. (n=3), *Fusarium solanii* (n=1) and *Rhyzopus oryzae* (n=1) were among fungal species isolated. According to our standard practice, cytomegalovirus (CMV) pp65 antigenaemia and Immediate Early Antigen detection by immunofluorescence (CINA kit, Argene Biosoft, Varilhes, France) and real-time PCR (COBAS Amplicor CMV Monitor test, Roche Diagnostics, Branchburg, NJ, USA) assays were routinely performed 1 or 2 times a week during the first 6 post-transplant months in CMV-seropositive recipients (Aversa et al., 2005). If any signs or symptoms were present, CMV disease was diagnosed.

Clinical evaluation. Clinical evaluation of fungal or viral infection was accomplished by clinical parameters as well as by computerized tomographic and magnetic resonance imaging diagnostics. Twenty five out of 39 patients developed fungal infections including pneumonia by *A. fumigatus* (n=18), *A. niger* (n=1) and *R. oryzae* (n=1); sinusitis by *A. fumigatus* (n=2), and *F. solanii* (n=1); invasive infection by *A. niger* (n=1) and encephalitis by *A. fumigatus* (n=1).

Genotyping of *TLR2*, *TLR4* and *TLR9* polymorphisms. Genotype determination of polymorphisms in *TLR2* (Arg677Trp and Arg753Gln), *TLR4* (Asp299Gly and Thr399Ile) and *TLR9* (T-1923C, T-1486C, T-1237C, G1174A and G2848A) were performed using bi-directional PCR amplification of specific alleles (Bi-PASA) (Carvalho et al., 2007). Primer design, PCR amplification and cycling were done as described (Carvalho et al., 2007; Carvalho et al., 2008).

The results obtained for the genotype of the study groups by Bi-PASA were further validated by sequencing analysis of 10 randomly selected samples.

Statistical analysis. The Fisher exact test and Pearson χ^2 test were used to compare allele frequencies between patient groups and controls. Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using a χ^2 test on a contingency table of observed vs. predicted genotype frequencies. χ^2 values, odds ratios, and p values were calculated with 95% confidence intervals.

RESULTS

To study the possible association of the different TLR polymorphisms with susceptibility to cytomegalovirus (CMV) infection, HSCT patients with and without clinical signs of viral infection were evaluated. Thirty three patients (50% of the studied group) showed signs of CMV infection that included pneumonia (n=24, 73% of infected patients), encephalitis (n=2, 6%), retinitis (n=1, 3%) and Epstein-Barr virus (EBV) co-infection (n=6, 18%). No statistically significant differences between patients with or without viral infection were observed regarding TLR polymorphisms (Table 1). However, patients with viral infection had a significantly higher frequency of the C allele on T-1237C (*TLR9*) than patients without infection (28.7% vs. 12.5%, respectively; p=0.02; OR, 2.83).

Patients with (n=39) and without (n=26) positive fungal cultures were assessed for possible association between fungal growth and *TLR2*, *TLR4* and *TLR9* polymorphisms. A comparison of allele frequencies and genotype distribution of the *TLR9* and *TLR2* polymorphisms revealed no statistically significant differences between patients with positive or negative fungal cultures. Although patients with positive fungal cultures had a higher frequency of the C allele on T-1486C (26.8% vs. 16.4%), allele A on G1174A (32.8% vs. 25.9%) and on G2848A (31.6% vs. 25.9%) regarding *TLR9* compared to patients with negative fungal cultures, these differences did not reach statistical significance (p=0.36 for C allele, and p=0.68 and p=0.80 for allele A of G1174A and G2848A, respectively) (Table 2). In contrast, patients with positive fungal cultures had a significantly higher frequency of allele G on Asp299Gly (*TLR4*) than patients with negative culture (12.8% vs. 2.0%, respectively; p=0.02; OR, 7.5) (Table 2).

Table 1. Genotype and allele frequencies of polymorphisms in *TLR9*, *TLR4* and *TLR2* genes in HSCT patients with and without clinical signs of viral infection.

Gene, SNP	Study groups (n)	Genotype n (%frequency)			Allele n (% frequency)		χ^2	p value	OR
TLR9									
A-1923C		**A/A**	**A/C**	**C/C**	**A**	**C**			
	No viral infection (32)	31	1	0 (0.0)	63 (98.5)	1 (1.5)			
	Viral infection (33)	30 (91.0)	3 (9.0)	0 (0.0)	63 (95.5)	3 (4.5)	0.97	0.32	3.00
T-1486C		**T/T**	**T/C**	**C/C**	**T**	**C**			
	No viral infection (32)	18	13	1	49 (77.5)	15 (22.5)			
	Viral infection (33)	24 (73.0)	8 (24.0)	1 (3.0)	56 (85.0)	10 (15.0)	1.44	0.23	0.58
T-1237C									
	No viral infection (32)	24 (75.0)	8 (25.0)	0 (0.0)	56 (87.5)	8 (12.5)			
	Viral infection (33)	16 (48.6)	**15 (45.4)**	2 (6.0)	47 (71.3)	**19 (28.7)**	5.24	0.02	2.83
G1174A		**G/G**	**G/A**	**A/A**	**G**	**A**			
	No viral infection (32)	16 (50.0)	16 (50.0)	0 (0.0)	48 (76.0)	16 (24.0)			
	Viral infection (33)	15 (46.0)	17 (51.0)	1 (3.0)	47 (71.5)	19 (28.5)	0.24	0.62	1.21
G2848A									
	No viral infection (32)	17 (53.5)	12 (37.2)	3 (9.3)	46 (73.0)	18(27.0)			
	Viral infection (33)	19 (58.0)	12 (36.0)	2 (6.0)	50 (75.8)	16 (24.2)	0.25	0.61	0.82
TLR4		**A/A**	**A/G**	**G/G**	**A**	**G**			
Asp299Gly									
	No viral infection (32)	25	7	0 (0.0)	57 (89.5)	7 (10.5)			
	Viral infection (33)	29 (88.0)	4 (12.0)	0 (0.0)	62 (94.0)	4 (6.0)	1.00	0.31	0.53
TLR2		**G/G**	**G/A**	**A/A**	**G**	**A**			
Arg677Trp									
	No viral infection (32)	32 (100.0)	0 (0.0)	0 (0.0)	64 (100.0)	0 (0.0)			
	Viral infection (33)	33 (100.0)	0 (0.0)	0 (0.0)	66 (100.0)	0 (0.0)	0.00	0.98	0.97
Arg753Gln									
	No viral infection (32)	32 (100.0)	0 (0.0)	0 (0.0)	64 (100.0)	0 (0.0)			
	Viral infection (33)	30 (91.0)	3 (9.0)	0 (0.0)	63 (95.5)	3 (4.5)	0.97	0.32	3.00

NOTE. OR, odds ratio; χ^2 and p values were calculated regarding allele frequencies.

Table 2. Genotype and allele frequencies of polymorphisms in *TLR9*, *TLR4* and *TLR2* genes in HSCT patients with negative or positive fungal cultures.

Gene, SNP	Study groups (n)	Genotype n (%frequency)			Allele n (% frequency)		χ^2	p value	OR
TLR9									
A-1923C		**A/A**	**A/C**	**C/C**	**A**	**C**			
	Negative fungal culture (26)	24 (92.4)	2 (7.6)	0 (0.0)	50 (95.0)	2 (5.0)			
	Positive fungal culture (39)	37 (95.0)	2 (5.0)	0 (0.0)	76 (98.8)	2 (1.2)	0.17	0.67	0.65
T-1486C		**T/T**	**T/C**	**C/C**	**T**	**C**			
	Negative fungal culture (26)	18 (69.6)	8 (30.4)	0 (0.0)	44 (83.6)	8 (16.4)			
	Positive fungal culture (39)	24 (62.5)	13 (32.5)	2 (5.0)	61 (73.2)	17 (26.8)	0.62	0.36	1.53
T-1237C									
	Negative fungal culture (26)	14 (62.0)	10 (30.4)	2 (7.6)	38 (72.2)	14 (27.8)			
	Positive fungal culture (39)	26 (67.5)	13 (32.5)	0 (0.0)	65 (78.0)	13 (22.0)	1.99	0.15	0.54
G1174A		**G/G**	**G/A**	**A/A**	**G**	**A**			
	Negative fungal culture (26)	14 (62.0)	11 (34.2)	1 (3.8)	39 (74.1)	13 (25.9)			
	Positive fungal culture (39)	17 (45.0)	22 (55.0)	0 (0.0)	56 (67.2)	22 (32.8)	0.16	0.68	1.18
G2848A									
	Negative fungal culture (26)	14 (62.0)	11 (34.2)	1 (3.8)	39 (74.1)	13 (25.9)			
	Positive fungal culture (39)	22 (57.5)	13 (32.5)	4 (10.0)	57 (68.4)	21 (31.6)	0.06	0.80	1.11
TLR4									
Asp299Gly		**A/A**	**A/G**	**G/G**	**A**	**G**			
	Negative fungal culture (26)	25 (96.1)	1 (3.9)	0 (0.0)	51 (98.0)	1 (2.0)			
	Positive fungal culture (39)	29 (74.3)	**10 (25.2)**	0 (0.0)	68 (88.0)	**10 (12.8)**	4.78	0.02	7.5
TLR2		**G/G**	**G/A**	**A/A**	**G**	**A**			
Arg677Trp									
	Negative fungal culture (26)	26 (100.0)	0 (0.0)	0 (0.0)	52 (100.0)	0 (0.0)			
	Positive fungal culture (39)	39 (100.0)	0 (0.0)	0 (0.0)	78 (100.0)	0 (0.0)	0.08	0.77	0.66
Arg753Gln									
	Negative fungal culture (26)	24 (92.4)	2 (7.6)	0 (0.0)	50 (95.0)	2 (5.0)			
	Positive fungal culture (39)	38 (97.5)	1 (2.5)	0 (0.0)	77 (92.4)	1 (8.0)	0.91	0.34	0.32

NOTE. OR, odds ratio; χ^2 and p values were calculated regarding allele frequencies.

Table 3. Genotype and allele frequencies of polymorphisms in *TLR9*, *TLR4* and *TLR2* genes in HSCT patients with or without clinical signs of fungal infection.

Gene, SNP	Study groups (n)	Genotype n (%frequency)			Allele n (% frequency)		χ^2	p value	OR
TLR9									
A-1923C		**A/A**	**A/C**	**C/C**	**A**	**C**			
	No fungal infection (14)	14 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)			
	Fungal infection (25)	23 (92.0)	2 (8.0)	0 (0.0)	48 (96.0)	2 (4.0)	0.53	0.46	2.25
T-1486C		**T/T**	**T/C**	**C/C**	**T**	**C**			
	No fungal infection (14)	7 (50.0)	7 (50.0)	0 (0.0)	21 (75.6)	7 (24.4)			
	Fungal infection (25)	17 (68.0)	6 (24.0)	2 (8.0)	40 (80.0)	10 (20.0)	0.26	0.60	0.75
T-1237C									
	No fungal infection (14)	9 (64.29	5 (35.8)	0 (0.0)	23 (82.2)	5 (18.7)			
	Fungal infection (25)	17 (68.0)	6 (32.0)	0 (0.0)	40 (84.0)	8 (16.0)	0.04	0.83	0.88
G1174A		**G/G**	**G/A**	**A/A**	**G**	**A**			
	No fungal infection (14)	7 (50.0)	7 (50.0)	0 (0.0)	21 (75.6)	7 (24.4)			
	Fungal infection (25)	10 (40.0)	15 (60.0)	0 (0.0)	35 (70.0)	15 (30.0)	0.22	0.63	1.29
G2848A									
	No fungal infection (14)	7 (50.0)	5 (35.5)	2 (14.5)	19 (67.9)	9 (32.1)			
	Fungal infection (25)	15	8 (32.0)	2 (8.0)	38 (76.0)	12 (24.0)	0.60	0.43	0.67
TLR4		**A/A**	**A/G**	**G/G**	**A**	**G**			
Asp299Gly									
	No fungal infection (14)	7 (50.0)	7 (50.0)	0 (0.0)	21 (75.6)	7 (24.4)			
	Fungal infection (25)	22 (88.0)	**3 (12.0)**	0 (0.0)	47 (94.0)	**3 (6.0)**	5.80	0.01	0.19
TLR2		**G/G**	**G/A**	**A/A**	**G**	**A**			
Arg677Trp									
	No fungal infection (14)	14 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)			
	Fungal infection (25)	25 (100.0)	0 (0.0)	0 (0.0)	50 (100.0)	0 (0.0)	0.18	0.67	0.55
Arg753Gln									
	No fungal infection (14)	14 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)			
	Fungal infection (25)	24 (96.0)	1 (4.0)	0 (0.0)	49 (98.0)	1 (2.0)	0.18	0.67	0.55

NOTE. OR, odds ratio; χ^2 and p values were calculated regarding allele frequencies.

Within the first month after transplant, 25 of the 39 patients with positive fungal culture developed fungal infections. No significant differences in the *TLR9* and *TLR2* polymorphisms between the two groups of patients (infected and non-infected) were observed. Even though a lower frequency of the A allele on G2848A (*TLR9*) was observed in patients with infection (24.0% vs. 32.1%, with and without infection, respectively), this difference did not reach statistical significance (p=0.43). Interestingly, the frequency of allele G on Asp299Gly SNP in *TLR4* was significantly lower in infected than non-infected patients (6.0% vs. 24.4%, respectively; p=0.01; OR, 0.19) (Table 3). Thus, the same *TLR4* polymorphism was associated with increased fungal colonization but decreased susceptibility to infection.

DISCUSSION

Given that TLR polymorphisms are associated with susceptibility to infection in a pathogen-specific manner (Schroder and Schumann, 2005; Schwartz and Cook, 2005), polymorphisms in these receptors could represent risk factors for infections in HSCT. The results of the present study show that distinct TLR SNPs are indeed associated with susceptibility to viral or fungal infection. TLR9 is critically required in the process of cytomegalovirus (CMV) sensing (Bozza et al., 2007; Tabeta et al., 2004) to assure rapid antiviral responses, coordinated with other TLR-dependent and –independent events that are required to establish adaptive immunity (Delale et al., 2005). We found that the C allele on T-1237C was highly predictive of susceptibility to CMV infection. As this SNP is associated with altered *TLR9* expression (Novak et al., 2007), our findings confirm the importance of the viral detection system for the activation of antiviral immune resistance. As a matter of fact, we have recently found that the exploitation of TLR9 as adjuvant receptor may provide new options for optimizing anti-CMV therapeutic strategies in experimental transplantation (Bozza et al., 2007).

Although the TLR9 SNPs tested tended to have a higher frequency in patients with positive fungal cultures and a lower frequency of the A allele on G2848A was observed in patients without infection, either the limited sample size might have reduced the power of the study, therefore increasing the chance for false-negative results, or TLR9 may be of secondary importance in fungal infections. In this regard, in addition to the fact that *A. nidulans* (and probably *A. fumigatus*) has been shown to be virtually devoid of genomic cytosine methylation

(Antequera et al., 1984), TLR9 has been found to exert control over allergy to the fungus more than infection (Bellocchio et al., 2004a; Grohmann et al., 2007). Accordingly, *TLR9* SNPs have been associated with an increased risk of asthma (Lazarus et al., 2003) and allergic bronchopulmonary aspergillosis (Carvalho et al., 2008), and TLR9 antagonists are under development for patients with asthma and other allergic conditions (Sun et al., 2007).

We observed no association between polymorphisms in *TLR2* and susceptibility to either viral or fungal infections, despite the fact that the Arg753Gln polymorphism has been associated with CMV replication and disease after liver transplantation (Kijpittayarit et al., 2007). In contrast, the results of the present study are similar to those obtained in haematopoietic transplanted patients with invasive aspergillosis (Kesh et al., 2005). As TLR2-deficient mice are highly susceptible to experimental ABPA (L. Romani, unpublished observations) more than *Aspergillus* pulmonary infection (Bellocchio et al., 2004a), our results are consistent with a crucial role for TLR2 in allergy (Vanhinsbergh et al., 2007) and lung inflammation (Dessing et al., 2008).

One interesting result of the present study concerns the intriguing role for TLR4 in the recognition and initiation of the inflammatory response to *Aspergillus*. Experimental evidence indicates TLR4 as one of the most important innate immune pathways in response to the fungus (Bellocchio et al., 2004a; Braedel et al., 2004; Chignard et al., 2007; Mambula et al., 2002; Netea et al., 2003). Accordingly, we have recently found that the Asp299Gly polymorphism is highly associated with chronic cavitary pulmonary aspergillosis (Carvalho et al., 2008). In the present study, we found a positive association between the Asp299Gly polymorphism and fungal colonization but not susceptibility to infection, confirming the previously observed lack of association of this SNP with aspergillosis in allogeneic stem cell transplantation (Kesh et al., 2005). The positive association of this SNP with fungal colonization could be explained by the fact that the presence of an abnormal TLR4 extracellular domain may hamper its function by disrupting microbial recognition, eventually leading to fungal escape from immunosurveillance. However, this polymorphism has also been shown to have a protective effect in other infectious diseases, such as malaria (Ferwerda et al., 2007), in hyper-inflammatory states, such as atherosclerosis (Li and Sun, 2007), and to be associated with longevity (Candore et al., 2006). Therefore, the failure to recognize the fungus may be compensated by the lack of an exuberant inflammatory response to it which may ultimately be harmful to the host. In this regard, we have recently found that a hyper-inflammatory state, more than the fungus itself, may contribute to susceptibility to aspergillosis and other fungal infections (Romani and Puccetti, 2007).

Paradoxically, the ability to restrain antifungal inflammation pointed to novel strategies for immune therapy that attempted to limit inflammation in order to stimulate an effective antifungal immune protection (Romani et al., 2008; Romani and Puccetti, 2007). Thus, by limiting the inflammatory response to the fungus, the Asp299Gly polymorphism could contribute to resistance to infection, despite evidence of fungal growth.

In candidiasis, resistance to infection with limited inflammatory response occurred upon the engagement of the Toll/IL-1 receptor (TIR) domain-containing adaptor protein inducing IFN (TRIF)-dependent pathway downstream TLR4 (De Luca et al., 2007). This resulted in the generation of "protective tolerance to the fungus", a homeostatic condition in which host immune defence mechanisms of protection are provided without necessarily eliminating fungal pathogens but with acceptable levels of inflammatory pathology and tissue damage (Romani and Puccetti, 2006). Whether and how the Asp299Gly polymorphism impairs fungal recognition and at the same time limits the host inflammatory response to it is presently under investigation. It is worth to anticipate here that, similar to candidiasis, the TRIF pathway also pivotally controls pathogenic inflammation to *Aspergillus* in experimental HSCT (L. Romani, unpublished observations).

Collectively, these results add to the current knowledge of the importance of TLRs in infections in HSCT. While CMV infection was significantly associated with altered *TLR9* expression, *Aspergillus* colonization but not infection was significantly associated with defective TLR4-dependent recognition, a finding suggesting that fungal infection and associated inflammatory disease may occur in a pathogen-autonomous manner. This will fit with the new emerging role of the innate immunity that is its role in sterile inflammation – inflammation caused by endogenous TLR ligand activation (Trinchieri and Sher, 2007). Therefore, despite that the sample size could not allow the precise appreciation of some associations, the results of the present study strongly point to the intricacies between pathogens and host immune responses in the setting of haematopoietic transplantation.

CHAPTER 6

Risk of developing non-Hodgkin lymphoma associates with the T-1237C polymorphism in the *TLR9* promoter

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ABSTRACT

Non-Hodgkin lymphoma (NHL) includes a set of heterogeneous lymphoproliferative malignancies often associated with altered immunological functions of the host and chronic inflammatory type of infections. Toll-like receptors, namely TLR9, has been shown to be associated with both autoimmune and chronic inflammatory type of diseases. Herein, we show that a polymorphism in the promoter region of *TLR9* – T-1237C – associates with an increased incidence of NHL. This polymorphism introduces a novel regulatory site that is *trans*-activated by the IL-6-dependent transcription factor IL-6 RE-BP, thus resulting in increased expression of *TLR9*. Cells harbouring the mutant allele have a higher expression of the *TLR9* gene, which is markedly increased upon receptor activation by ligand binding. TLR9 activation in B lymphocytes sequentially leads to increased proliferation rates of these cells, as well as higher production of IL-6, resulting in a TLR9 activation loop leading to B lymphocyte-specific uncontrolled proliferation. Taken together, our observations suggest that the T-1237C polymorphism confers host predisposition to NHL by increased cellular proliferation, making B lymphocytes more susceptible to acquire transforming mutations associated with the development of NHL. Our data, showing that the T-1237C polymorphism in the *TLR9* promoter leads to an increased *TLR9* expression under the influence of the pro-inflammatory cytokine IL-6, has important implications on the usage of CpG agonists on several therapeutic strategies.

INTRODUCTION

Non-Hodgkin lymphoma (NHL) is a haematological disease responsible for a large number of cancer-related deaths. NHL includes a heterogeneous group of malignant lymphoproliferative diseases whose incidence has substantially increased over the past decades in western countries (Parkin, 2001). Genetic and environmental factors underlying an altered immunological function are the best characterized and known risk factors for NHL, although its aetiology and the underlying causes for the observed increase are largely unknown (Baris and Zahm, 2000). Individuals with immune deficiencies associated with immune suppressive therapy after transplantation, human immunodeficiency virus (HIV) infection and congenital conditions are among those with higher incidence rates of lymphoid malignancies (Beral et al., 1991; Filipovich et al., 1992). Additionally, clinical and experimental data consistently associated several autoimmune and chronic inflammatory disorders, including rheumatoid arthritis and systemic lupus erythematosus, with an increased risk of NHL (Grulich et al., 2007). Some NHL subtypes have also been linked with specific infectious agents including *Helicobacter pylori* and several viruses, namely Epstein-Barr virus (Muller et al., 2005).

Although the immunological status of the host is considered one of the major determinants in NHL development, not much is known about this subject. A recent large scale evaluation of polymorphisms in immune and inflammatory genes reinforced the idea that variations in genes related to immune function may predispose to the development of NHL (Cerhan et al., 2007). Genetic variants in tumour necrosis factor (*TNF*) and interleukin-10 (*IL10*), key cytokines involved in the inflammatory response and immune balance, were shown to increase the risk of NHL and influence the clinical course of the disease (Bogunia-Kubik et al., 2008; Rothman et al., 2006). In addition, experimental evidence also supports a role for variants in cytotoxic T lymphocyte antigen 4 (*CTLA4*) in susceptibility to NHL (Monne et al., 2004; Piras et al., 2005). More recently, a functional variant in the gene encoding for the costimulatory molecule CD40, associated with reduced circulating levels of the protein and lower CD40 surface expression by dendritic cells, was also proposed as risk factor for non-Hodgkin lymphoma, in particular follicular lymphoma (Skibola et al., 2008).

However, in recent years, considerable attention has focused on the importance of innate immunity, the first line of defence which differentiates self from non-self and activates the adaptive immune system through specific signals (Fearon and Locksley, 1996; Medzhitov and

Janeway, 1997). The innate immune system is able to recognize conserved molecular motifs in pathogens through pattern recognition receptors, including Toll-like receptors (TLRs). TLRs mediate the recognition of microbial challenges and the subsequent inflammatory responses through rapid changes in the expression of genes encoding cytokines and inflammatory molecules (Akira et al., 2001). Several genetic variants in TLRs have already been characterized regarding susceptibility to infectious and inflammatory diseases (Schroder and Schumann, 2005). The first report linking TLRs and susceptibility to cancer showed that a polymorphism in TLR4 - Asp299Gly - predisposed to gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Hellmig et al., 2005). However, the extent to which genetic variants in TLRs increase the risk of NHL is still unknown.

TLR-mediated recognition of microbial stimuli often leads to the activation of innate immune cells, including B lymphocytes (Akira et al., 2001; Iwasaki and Medzhitov, 2004). These cells are known to have a TLR repertoire in which TLR9, a receptor that detects unmethylated CpG motifs prevalent in bacterial and viral DNA, predominates (Bourke et al., 2003; Hemmi et al., 2000). The T-1237C polymorphism, located within the putative promoter region of the *TLR9* gene, has already been shown to predispose to several autoimmune and chronic inflammatory conditions, including asthma, Crohn's disease and systemic lupus erythematosus (Demirci et al., 2007; Lazarus et al., 2003; Torok et al., 2004). Additionally, viral infections and their persistent antigen-driven immune receptor activation also support a role for chronic stimulation in the clonal evolution of virus-associated NHL (Ivanovski et al., 1998). The involvement of TLR9, not only in chronic inflammatory disorders, but also in viral recognition, suggests that the T-1237C polymorphism may have a major impact in NHL, often concurrent with the development of chronic inflammatory pathologies as well as viral infection.

Taking all these observations into account, we hypothesized that the presence of the T-1237C polymorphism in the *TLR9* gene could confer susceptibility to NHL. We show that the polymorphism associates with NHL, by introducing a new regulatory site responsive to an IL-6-dependent transcription factor.

MATERIALS AND METHODS

Study populations. The case population consisted of 252 non-Hodgkin lymphoma (NHL) patients (mean age=55.0±16.2; 55.6% males, 44.4% females) of Portuguese origin. These patients were recruited from hospitals and office-based physicians involved in the diagnosis and treatment of lymphoma. For 147 patients, the disease was classified in its subtypes; for subtype-specific analysis, only follicular lymphoma, FL (n=68, 46.3%), and diffuse large B cell lymphoma, DLBCL (n=35, 23.8%), were considered. Patients who had a history of transplantation or human immunodeficiency virus (HIV) infection were excluded from the study. The control population consisted of 789 unrelated healthy blood donors frequency-matched to patients by age within 5 years, sex and country of origin. Study protocols were approved by each institution's ethic review committee. All study participants provided written informed consent prior to biospecimen collection.

Determination of T-1237C genotype. Genomic DNA was extracted from leucocytes with the NucleoSpin Blood DNA isolation kit (Macherey-Nagel, Düren, Germany). Determination of the T-1237C genotype (SNP id: rs5743836) was performed using bi-directional PCR amplification of specific alleles (Bi-PASA), as previously described (Carvalho et al., 2007).

Analysis of *TLR9* promoter region. The promoter sequence in the vicinity of the T-1237C polymorphism site was analyzed in order to predict and compare potential transcription factor binding sites in relation to the genotype at the -1237 nucleotide position. Analysis was performed in 100% matrix to sequence homology by using the Transcription Element Search System (TESS) interface. The parameters used in the prediction of transcription factor binding motifs included L_a, the log-likelihood score, and L_q, a measure of the goodness-of-fit of the DNA sequence to the consensus binding motif, calculated by dividing L_a by the maximum L_a possible for the TESS interface; the best possible L_q value was 1.000.

Plasmid constructs and mutagenesis. The plasmid vector containing the luciferase gene under the control of the wild-type *TLR9* promoter (pGL3-hTLR9-T) was a generous gift from Dr. Fumihiko Takeshita (Takeshita et al., 2004). The plasmid was used to perform site-directed PCR mutagenesis. Briefly, the single nucleotide substitution at position -1237 in the 5'-flanking

region was introduced by PCR amplification using the forward primer 5'-TATGAGACTTGGGGGAGTTTCCAGGCAGAGGGAACAGCACA-3' and the reverse primer 5'-TGTGCTGTTCCCTCTGCCTGGAAACTCCCCCAAGTCTCATA-3'. An all-round PCR amplification was performed in a 20 µl volume that included autoclaved ultra-filtered water, 1× PCR buffer, 200 µM dNTP mixture, 2.5 µM primers, 2.5 U of *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) and approximately 200 ng of plasmid template. The PCR product was then digested with *DpnI*, removing methylated parental template, and cloned in *Escherichia coli*. Transformants carrying the plasmid vector with the luciferase gene under the control of the mutant *TLR9* promoter (pGL3-hTLR9-C) were selected using Bi-PASA (Carvalho et al., 2007). The promoter region of selected transformants was then amplified and sequenced to confirm the correct base substitution.

Cell lines and cell culture. The Raji cell line (human B lymphocytes derived from Burkitt's lymphoma) was purchased from ATCC (American Type Culture Collection, Manassas, VA). Cells were cultivated in RPMI culture medium [RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% fetal bovine serum (FBS)] without antibiotics at 37°C and 5% CO_2. The medium was renewed every 2-3 days.

Transient transfection and luciferase reporter gene assay. Raji cells (5×10^6 cells/ml) cultured in RPMI medium were transfected using the Microporator system (NanoEnTek Inc, Seoul, Korea) with a 10 µl tip (pulse voltage of 1350 V and width of 30 ms) with 0.5 µg of the plasmid constructs. To normalize transfection efficiency, 0.5 µg of the control β-galactosidase vector (pCMV-βgal) were always co-transfected. Cells were then resuspended in antibiotic-free RPMI medium, seeded in 48-well plates and incubated for 24 hours. After recovery, human recombinant IL-6 was added to 1×10^6 cells (100 ng/µl) and cells were cultured for 12 hours. Cells were then lysed in 200 µl of 1× Passive Lysis Buffer (Promega, Madison, WI, USA) and luciferase activity in 10 µl aliquots of the cell lysates was measured using the Luciferase Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized against β-galactosidase activity, which was determined with the β-galactosidase Enzyme Assay System (Promega), according to the manufacturer's instructions. Data are expressed in relative luciferase units, with the unit corresponding to the normalized luciferase

activity. All experiments were performed in triplicate and repeated at least in two different occasions.

Peripheral blood mononuclear cell (PBMC) culture. PBMC were isolated using Histopaque-1077 (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. After centrifugation, cells were resuspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.05% EDTA, and counted in a hemocytometer. The viability of cells was determined using trypan blue exclusion. Aliquots of 200 µl containing 1×10^6 PBMC were then seeded in 48-well flat bottom plates and cells were cultivated for 5 days in the presence of 0.05 µM CpG ODN 2006 (5'-tcgtcgttttgtcgttttgtcgtt-3') (InvivoGen, Toulouse, France) at 37°C in a 5% CO_2 humidified atmosphere. Cells either unstimulated or stimulated with control CpG ODN 2006 (5'-tgctgcttttgtgcttttgtgctt-3') (InvivoGen) were included as controls throughout the study.

CFSE proliferation assay. PBMC were resuspended at a concentration of 2.5×10^6 cells/ml in serum-free Hank's Balanced Salt Solution (HBSS) and labelled with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) at a final concentration of 5 µM for 5 min at room temperature with occasional shaking. Labelling was quenched by the addition of 1/5 of the total volume of FBS 10%. Cells were then washed twice with in complete RPMI medium and resuspended at the concentration of 2×10^6 cells/ml before culturing in 24-well plates. Next, PBMC stimulated with CpG ODN for 5 days at 37°C and 5% CO_2 were washed, resuspended in staining buffer (0.5% BSA, 0.04% EDTA, 0.05% sodium azide in PBS) and stained with APC-conjugated anti-human CD19 (Becton Dickinson, San Jose, CA, USA) for 15 min at room temperature in the dark and analyzed on a BD FACSCalibur flow cytometer (Becton Dickinson).

Real-time RT-PCR. After the 5-day CpG ODN stimulation described above, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Marnes La Coquette, France). For RT-PCR, 1 µl of cDNA was used as template in a 20 µl reaction conducted using the QuantiTect Sybr Green PCR Kit (Qiagen) according to manufacturer's instructions. Each sample was measured in triplicate. The RT-PCR primers used were as follows: β-actin, sense: 5'-GCCGTCTTCCCCTCCATCGTG-3', antisense: 5'-

GGAGCCACACGCAGCTCATTGTAGA-3'; TLR9, sense: 5'-CAGCAGCTCTGCAGTACGTC-3', antisense: 5'-CTCAGGCCTTGGAAGAAGTG-3'. RT-PCR was conducted on a LightCycler System (Roche Applied Science, Basel, Switzerland) and the thermal cycling conditions included 50 cycles of 10 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C, after a 15-min initial step of enzyme activation at 95 °C, a melting step of 55-95 °C (0.5 °C/s) and a final cooling step at 40 °C. Expression of β-actin was used as an internal control for all of the samples. Expression of *TLR9* is indicated as n-fold increase relative to the level of *TLR9* expression in untreated wild-type cells using the $2^{\Delta\Delta_{Ct}}$ method, as described previously (Livak and Schmittgen, 2001).

IL-6 quantification. IL-6 present in supernatants from PBMC cultures either in untreated conditions or stimulated for 5 days with CpG ODN 2006 was quantified using human IL-6 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

Statistical analysis. Patients were analysed as all NHL and by the two major subtypes, FL and DLBCL. Fisher's exact test and Pearson's χ^2 test were used to compare genotype frequencies between the patient groups and controls. Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using a χ^2 test on a contingency table of observed versus predicted genotype frequencies. The values of χ^2, odds ratio (OR) and p were calculated at 95% confidence intervals (CI). Analyses were conducted using the SPSS 16.0 software.

RESULTS

The T-1237C polymorphism associates with risk of developing non-Hodgkin lymphoma (NHL). To determine whether there was an association of the T-1237C polymorphism with NHL, we analysed a population of 252 patients with different subtypes of the disease, the most frequent being follicular lymphoma (FL, n=68) and diffuse large B cell lymphoma (DLBCL, n=35). Genotype frequencies of control individuals (n=789) for the T-1237C polymorphism were in Hardy-Weinberg equilibrium ($p>0.05$).

A comparison of genotype distribution of the T-1237C polymorphism between the controls and the total number of NHL patients revealed a significant association between the

presence of the C allele and development of this haematological malignancy (19.4% vs. 31.0%; p<0.001; odds ratio [OR], 1.86; 95% confidence interval (CI), 1.35-2.57) (Table 1). Moreover, this association occurs independently of the major NHL subtypes considered, FL (19.4% vs. 30.9%; p<0.05; OR, 1.86, CI, 1.08-3.18) and DLBCL (19.4% vs. 37.1%; p<0.05; OR, 2.46, CI, 1.22-4.93). No significant associations were found when considering other features of clinical value including international prognostic indexes (IPI and FLIPI), clinical stage of the disease and overall survival (data not shown).

Table 1. Genotype distribution of the T-1237C polymorphism in the total number of non-Hodgkin lymphoma (NHL) patients, follicular lymphoma (FL) or diffuse large B cell lymphoma (DLBCL) subtypes, and controls

SNP	Study groups (n)	Genotype n (% frequency)				χ^2	p value	OR (95% CI)*
		T/T	T/C	C/C	T/C + C/C			
TLR9 T-1237C	Controls (789)	636 (80.6)	144 (18.3)	9 (1.1)	153 (19.4)			
	All NHL (252)	174 (69.0)	76 (30.2)	2 (0.8)	**78 (31.0)**	14.8	0.000	1.863 (1.354-2.565)
	FL (68)	47 (69.1)	21 (30.9)	0 (0.0)	**21 (30.9)**	5.11	0.028	1.857 (1.084-3.184)
	DLBCL (35)	22 (62.9)	13 (37.1)	0 (0.0)	**13 (37.1)**	6.56	0.016	2.456 (1.225-4.929)

* Odds ratio (OR), confidence interval (CI). χ^2 and p values were calculated regarding presence of variant allele.

***TLR9* promoter activity is altered by the T-1237C polymorphism.** The base substitution at nucleotide position -1237 was assessed for predicted effects on transcription factor-binding motifs. An *in silico* analysis of the *TLR9* promoter using the TESS interface showed that the T-1237C polymorphism introduces a new potential regulatory transcription factor-binding motif for interleukin-6 (IL-6) response element (IL-6 RE) at position -1238 to -1234 with the consensus sequence TTCCAG. This IL-6 RE is a binding site for, and is *trans*-activated by the IL-6-inducible IL-6 RE-binding protein (IL-6 RE-BP), suggesting an altered role of IL-6 in the regulation of the *TLR9* gene in individuals harbouring this polymorphism.

To explore the effect of the C allele on *TLR9* gene expression in B lymphocytes, 3.2 kb of both the wild-type and the mutant human promoter sequence were placed upstream of a

luciferase reporter gene and the constructs were transiently expressed in human Raji B cells. Transient transfection assays with the use of these promoter-reporter constructs showed significantly lower transcriptional activity for the C allele construct in basal conditions ($p<0.001$) (Figure 1A). In contrast, recombinant human IL-6 stimulation increased the transcriptional efficiency for the mutant C allele by 3-fold as compared with the T allele ($p<0.001$). Moreover, the allele-specific transcription data also showed that the ratio of IL-6-stimulated to basal gene expression in the C allele was more than three times that in the T allele ($p<0.001$). These results indicate that the T-1237C polymorphism has a major influence on the transcriptional regulation of the *TLR9* gene.

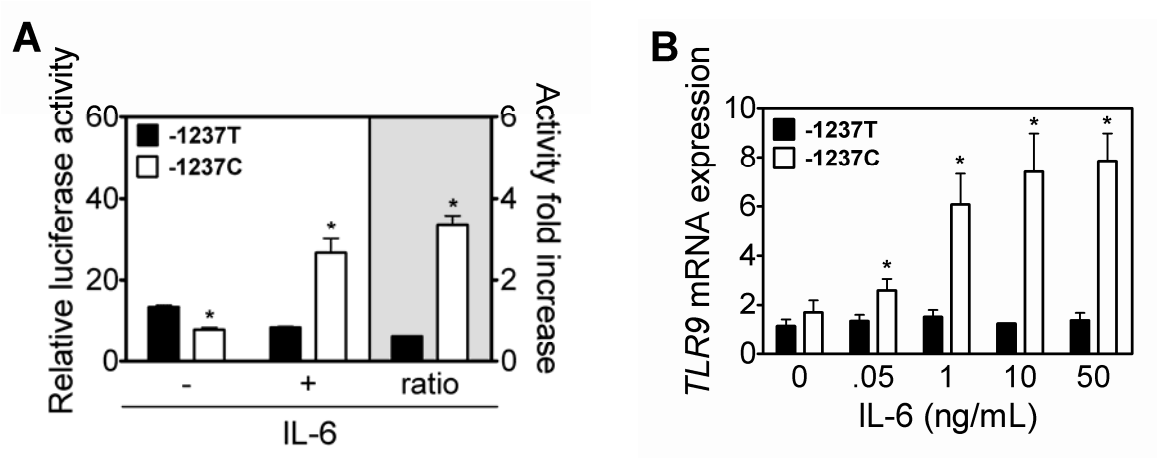

Figure 1. IL-6 induces an increase in *TLR9* expression in cells harbouring the mutant allele of the T-1237C polymorphism. **A.** Luciferase reporter assay of human Raji B cells transfected with plasmid vectors containing the luciferase gene under the control of the wild-type or mutant *TLR9* promoter. Cells (1×10^6 cells/ml) were transfected by electroporation and stimulated with IL-6 (100 ng/ml) for 12 h. Results shown are the means of relative luciferase activity units corrected to the transfection efficiency ± SD of at least three independent transfections **B.** Dose-dependent stimulation of human PBMCs with different T-1237C genotypes by IL-6. Human PBMCs (1×10^6 cells/ml) were stimulated with IL-6 over the concentration range from 0 (addition of a neutralizing anti-IL-6 antibody) to 100 ng/ml. Cells were harvested at 12 h and total RNA was isolated to determine *TLR9* mRNA expression levels. Results shown are the means ± SD of at least six independent observations.

IL-6 promotes an increase in *TLR9* expression in cells harbouring the C allele. To analyze the previously observed effects of the T-1237C, peripheral blood mononuclear cells (PBMCs) were isolated from unrelated healthy blood donors with different T-1237C

genotypes and treated with increasing concentrations of recombinant human IL-6. Quantification of *TLR9* mRNA expression showed that, in wild-type cells, no changes in *TLR9* expression were observed either upon stimulation with different IL-6 concentrations or in the total absence of endogenous IL-6, achieved using a neutralizing anti-IL-6 antibody (Figure 1B). In contrast, cells carrying the C allele responded to the presence of IL-6 with an increased expression of *TLR9* (Figure 1B). For the higher IL-6 concentrations tested, *TLR9* expression values became very similar, suggesting a saturation-like behaviour in the response to IL-6.

In normal conditions, with no addition of exogenous IL-6, nor neutralization of IL-6, cells carrying the C allele displayed an approximately 4-fold increase in basal transcript amount of *TLR9* as compared with the T allele (Figure 2A). This higher basal expression of *TLR9* was dependent on IL-6, as the addition of neutralizing anti-IL-6 antibody decreased the *TLR9* expression in cells with the C allele to levels similar to those displayed by their wild-type counterparts.

We also wanted to investigate whether the natural ligand for TLR9 would modulate its expression and this modulation was different in wild-type or mutant cells. We observed that TLR9 activation through CpG stimulation induced a strong increase in *TLR9* expression in cells with the C allele, reaching more than a 5-fold increase as compared to wild-type cells (Figure 2A) ($p<0.005$). No significant differences in gene expression were observed in CpG ODN 2006-stimulated cells with the wild-type genotype. Importantly, the CpG induction of *TLR9* mRNA in mutant cells was dependent on IL-6, as the neutralization of this molecule abrogated the effect of IL-6. Simultaneous stimulation of cells with CpG ODN 2006 and the TLR9 antagonist CpG TTAGGG in a 1:2 ratio did not result in any variation in *TLR9* expression from that displayed in the control conditions ($p<0.005$) (Figure 2A).

The C allele underlies increased proliferation upon TLR9 activation. To evaluate whether the T-1237C polymorphism could affect CpG ODN 2006-induced B cell expansion *in vitro*, PBMCs with different T-1237C genotypes were CpG ODN 2006-stimulated and proliferation of $CD19^+$ cells was assessed by monitoring the dilution of carboxyfluorescein succinimidyl ester (CFSE). CpG ODN 2006 concentrations ranging from 0.01 μM to 3 μM were tested and the optimal concentration of 0.05 μM was used (data not shown).

Although a *TLR9* overexpression was observed in untreated cells with the C allele, proliferation was not observed in untreated conditions for either genotype (Figure 2B). However,

in CpG ODN 2006-stimulated conditions, while wild-type $CD19^+$ cell proliferation was moderately induced by CpG ODN (32.5% $CFSE_{low}$ cells), the proliferation was significantly higher in cells with the C allele (64.3%) ($p<0.001$). Stimulation with a control CpG ODN 2006 (without the normal stimulatory sequence) led to reduced proliferation, although always higher in cells harbouring the C allele (Figure 2B).

The increase in the proliferation of $CD19^+$ cells harbouring the C allele upon TLR9 activation was shown to be IL-6-dependent since the addition of a neutralizing anti-IL-6 antibody was able to abrogate the effect of CpG ODN 2006 stimulation on cells carrying the C allele (Figure 2B). Moreover, increased proliferation displayed by cells with the mutant allele upon TLR9 activation was also demonstrated as being TLR9-specific since simultaneous stimulation with CpG ODN 2006 and a known TLR9 antagonist (TTAGGG ODN) reverted proliferation to basal values for both alleles (Figure 2B).

Cells harbouring the C allele display higher IL-6 production upon TLR9 activation. To assess if the production of IL-6, one of the cytokines more abundantly secreted after TLR9 activation in B lymphocytes, was dependent on the T-1237C polymorphism, IL-6 was quantified in supernatants from PBMC cultures after 5-day stimulation with CpG ODN 2006 (Figure 2C). The results show that CpG ODN 2006 stimulation resulted in a substantially higher IL-6 production by cells harbouring the C allele (156.4±49.4 pg/ml) as compared with cells carrying the wild-type promoter (44.2±17.0) ($p<0.05$), suggesting a direct correlation of the IL-6 amount and cellular proliferation displayed by cells with the mutant allele (Figure 2C).

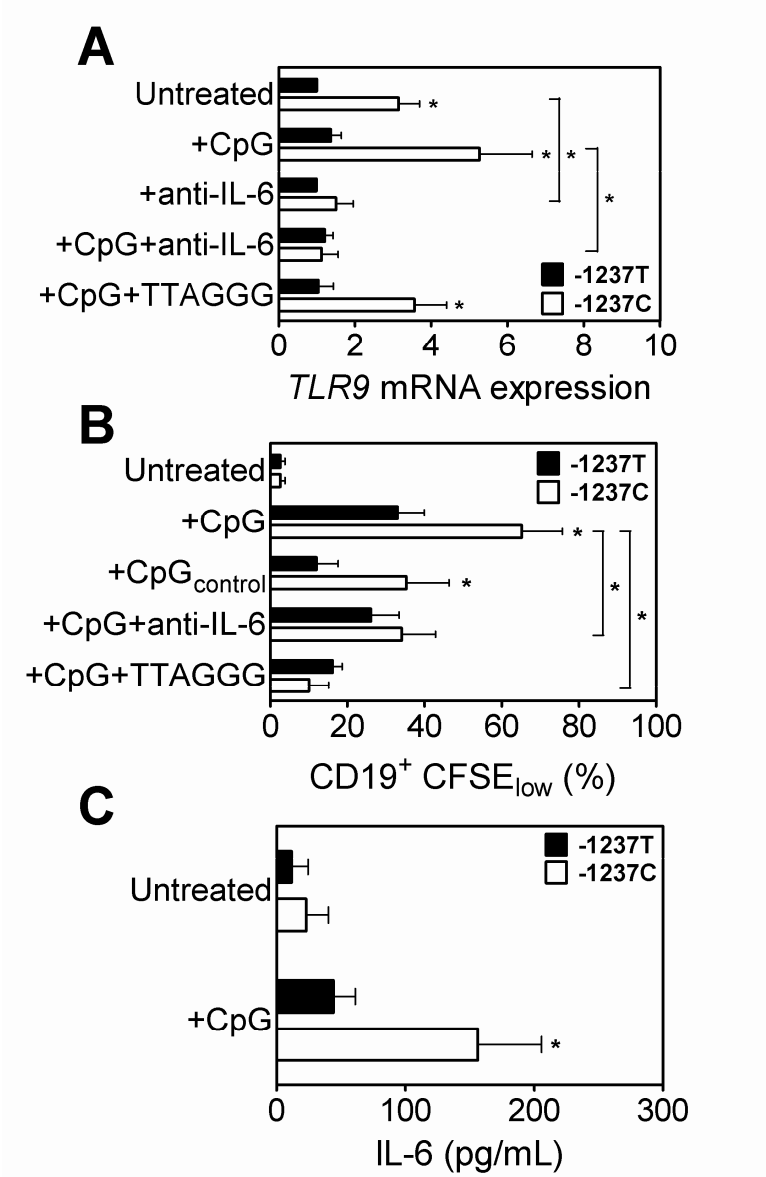

Figure 2. TLR9 activation of cells harbouring the mutant allele of the T-1237C polymorphism by CpG ODN 2006 increases *TLR9* gene expression, B cell proliferation and IL-6 secretion. **A.** *TLR9* mRNA expression of human PBMCs with different T-1237C genotypes (1×10^6 cells/ml) treated in the following conditions for 5 days: untreated and stimulated with either CpG ODN 2006 (0.05 μM), anti-IL-6 antibody (1:10), CpG ODN 2006 and anti-IL-6 antibody, or CpG ODN 2006 together with the TLR9 antagonist CpG ODN TTAGGG in a 1:2 ratio. Results shown are the means ± SD of at least six independent observations. **B.** Proliferation of $CD19^+$ cells assessed by CFSE dilution in cultures of human PBMCs with different T-1237C genotypes (1×10^6 cells/ml) after treatment in the following conditions for 5 days: untreated and stimulated with either CpG ODN 2006 (0.05 μM), CpG ODN 2006 control (0.05 μM), CpG ODN 2006 and anti-IL-6 antibody (1:10) or CpG ODN 2006 and CpG ODN TTAGGG in a 1:2 ratio. Results shown are the means ± SD of at least twelve independent observations. **C.** Quantification of IL-6 in supernatants of cultures of human PBMCs with different T-1237C genotypes (1×10^6 cells/ml) either in untreated conditions or stimulated with CpG ODN 2006 (0.05 μM) for 5 days. Results shown are the means ± SD of at least six independent observations.

DISCUSSION

Host genetic factors are emerging as key determinants of susceptibility to diseases of diverse aetiologies, including cancer. Identifying candidate genes is a major challenge that has to stem from a profound understanding of the pathophysiology of the disease. Recently, considerable attention has been given to genetic variations of genes of the innate immune system. While variations on these genes may be advantageous at the population level, allowing for a more intricate repertoire and enabling the host to withstand microbial challenges, there may be less favourable outcomes for individuals that harbour certain genotypes associated with excessive immune activation and inflammatory drive.

TLRs are differentially expressed on a wide variety of tumours (Bourke et al., 2003), suggesting that they may play important roles in cancer biology. In fact, dynamic interaction between malignant cells and the innate immune system is essential for tumour survival, growth and metastasis (Yu and Chen, 2008). However, cancer cells are also under surveillance due to their recognition by immune cells as foreign, thus having to overcome such immune surveillance in order to progress. The importance of TLRs in cancer immunity is evident in an increasing number of reports showing that variants in these receptors are associated with cancer risk. More specifically, the aetiology of some lymphoma subtypes has been associated with polymorphisms in *TLR1*, *-2*, *-4*, *-5* and *-9* genes (Nieters et al., 2006). Our data clearly show an association between the T-1237C polymorphism in the promoter region of *TLR9* and non-Hodgkin lymphoma (NHL), increasing the risk by more than 80% independently of the lymphoma subtype.

The genetics of haematological malignancies in general is complex. Although there is often a well-recognized association between the immunodeficient status of the host and susceptibility to NHL, little is known about the cellular and molecular mechanisms leading to abnormal lymphocyte proliferation or how they are linked to the development of a particular NHL subtype. Our study indicates that TLR9, through its biological effects (e.g. cell survival, proliferation, and inflammation), may be contributing to the pathogenesis of NHL in a cell-specific manner. In this way, a deregulation of *TLR9* expression and activity is suggested to directly contribute to the development of NHL through the induction of B lymphocyte-specific uncontrolled proliferation (Figure 3). In addition, the effect of the T-1237C polymorphism would not contribute to disease manifestation unless an underlying chronic stimulation of the immune system leading to a persistent activation of TLR9 was present.

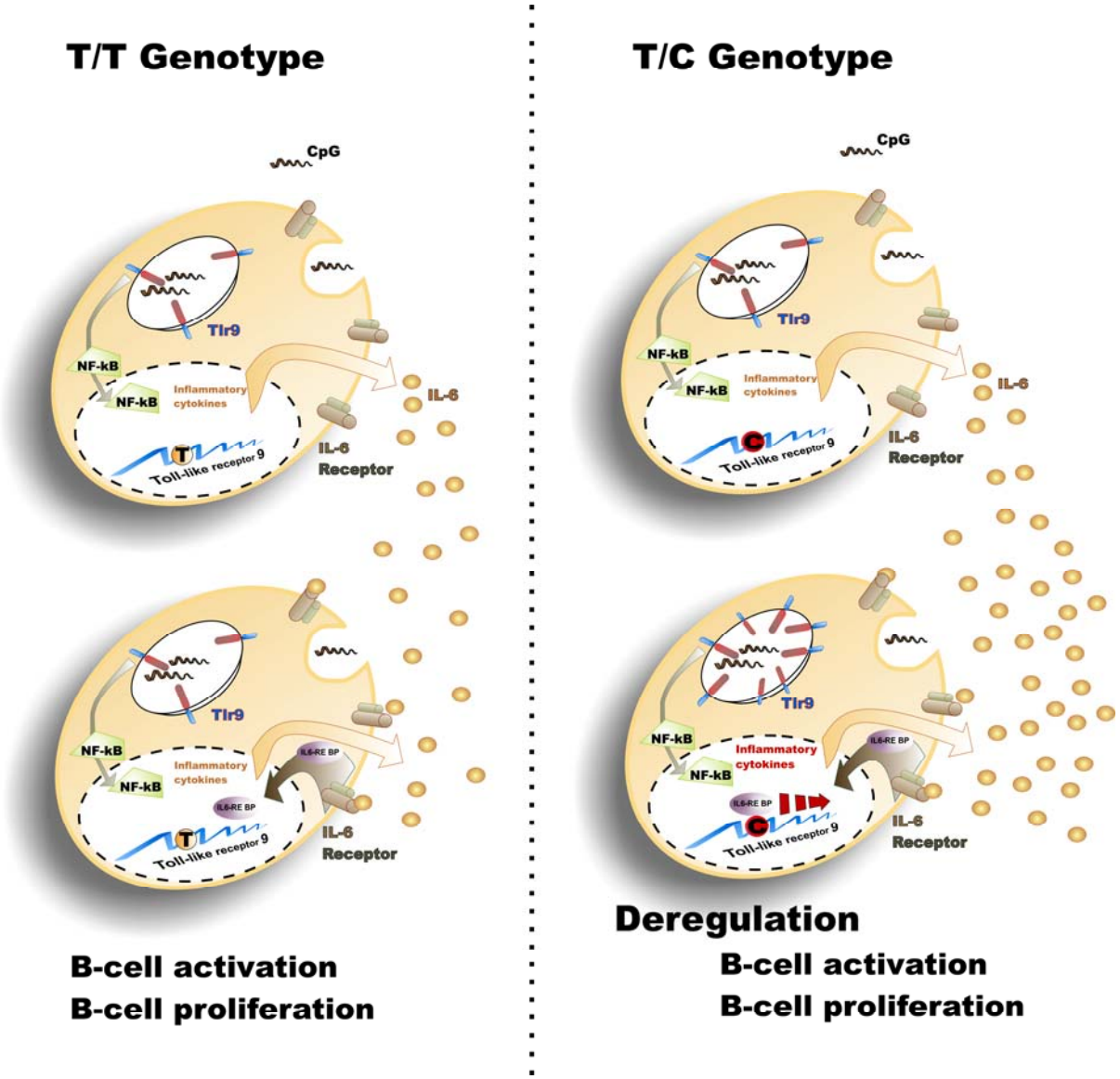

Figure 3. Proposed model representing the effect of the T-1237C polymorphism in B lymphocyte activation and proliferation. TLR9 activation by its ligand, CpG DNA, induces the production of IL-6, which binding to its receptor, promotes translocation of the IL-6 response element binding protein (IL-6 RE-BP) to the nucleus. In cells harbouring the variant allele, the transcription factor will bind the IL-6 RE and increase *TLR9* expression and consequently the number of receptor molecules. Upon TLR9 activation, these cells will produce abnormally high concentrations of IL-6, resulting in a loop of signal amplification that leads to a deregulation in cell activation and proliferation.

We have shown that the presence of allele C at position -1237 alters the transcriptional regulation of the *TLR9* gene and that binding of the IL-6 RE-BP transcription factor up-regulates *TLR9* promoter activity, suggesting a pivotal role for IL-6 in the mechanism underlying increased susceptibility to NHL. In fact, IL-6 is known to activate signal transducer and activator of

transcription 3 (STAT3) (Akira et al., 1994), whose constitutive activation is observed in many types of tumours, promoting cell survival and proliferation (Haura et al., 2005). Our results showed that cells harbouring the C allele, presumably expressing more TLR9 molecules than its wild-type counterpart, also proliferated more in response to the receptor activation. These results suggest that a constant activation of TLR9 by persistent ligand binding, such as that occurring in chronic inflammatory diseases, could lead to increased proliferation rates of B lymphocytes. This proliferation would be considerably amplified in individuals carrying the mutant allele, a premise also supported by the fact that the aforementioned persistent infections would lead to increased levels of circulating IL-6, thus resulting in augmented *TLR9* expression and ultimately increased amounts of receptor molecules. On the other hand, TLR9 activation in B lymphocytes, especially those harbouring the C allele, leads to increased release of IL-6, thus adding up to the effect and promoting a loop of signal amplification. The exacerbated cellular proliferation resulting from chronic immune stimulation by TLR9 ligands would eventually make B lymphocytes more susceptible to acquire transforming mutations, such as *bcl-2* translocations or mutations affecting the *c-myc* gene (Ivanovski et al., 1998; Pagano, 2002; Staak et al., 2002).

The neoplastic process leading to the development of NHL may be usurping impairments in TLR signalling pathways to advance cancer progression, which suggests that targeting these pathways may open novel therapeutic avenues. CpG oligodeoxynucleotides (ODNs) are now regarded as highly promising for the use in cancer therapy, mostly due to the direct effect of TLR9 activation on immune cell subpopulations that play an important role in anti-tumour immunity, including B cells (Krieg et al., 1995). CpG ODNs are also known to induce enhanced production of a number of cytokines with anti-tumour activity, including TNF, IL-12 and IFN-γ (Lipford et al., 1997). However, therapeutic applications of these CpG ODNs should be individually tailored since, as shown in the present study, individual variations can affect the outcome of the TLR9 signalling pathway. Our results showed that simultaneous stimulation of cells harbouring the mutant allele of T-1237C with both CpG ODN and CpG TTAGGG, a known TLR9 antagonist, reverted the elevated proliferation rates displayed by these cells when solely stimulated with CpG ODN. In this sense, our results not only discard the use of stimulatory CpG ODNs in this particular subset of NHL patients, but suggest a potential application of TLR9 antagonists in the suppression of the uncontrolled cellular proliferation that may be underlying the NHL risk in patients harbouring the mutant allele of T-1237C.

In many chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus, IL-6 signalling is critically involved in the maintenance of a disease state by promoting transition from acute to chronic inflammation (Rose-John et al., 2006), which often correlates with the development of NHL. Accordingly, our results have shown that IL-6 also plays a crucial role in the increased *TLR9* expression exhibited by cells carrying the mutant allele of T-1237C. However, the resulting increased proliferation can be abrogated by the use of a neutralizing anti-IL-6 antibody, which similarly to CpG TTAGGG, is able to suppress proliferation after TLR9 stimulation to values close to those displayed by the wild-type cells. This, as occurred for the TLR9 antagonist, suggests a potential application of anti-IL-6 antibodies in controlling not only inflammation but aberrant proliferation, at least in this particular scenario.

In conclusion, our study demonstrates an association between the T-1237C polymorphism and susceptibility to NHL and suggests that increased B lymphocyte proliferation resulting from an amplified *TLR9* expression due to an IL-6-dependent *trans*-activation may be involved in the mechanism underlying individual susceptibility to the development of this particular lymphoproliferative malignancy.

CHAPTER 7

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Fungi are ubiquitous organisms in the environment that are constantly interacting with the human host. Despite their abundance and widespread distribution, most of the fungal species do not trigger disease in healthy individuals; however some act as opportunistic pathogens in individuals with specific immune defects. Invasive, life-threatening fungal infections are undoubtedly the most important group of fungal infections, whose vast majority is attributable to *Candida* and *Aspergillus* species. The incidence of these infections has risen in the past years mostly due to advanced medical and surgical interventions that compromise patient immunity, including immunosuppressive therapy producing prolonged neutropenia, such as that occurring in stem cell or solid-organ transplanted patients (Fridkin and Jarvis, 1996). In addition, despite the development and availability of new antifungal drugs, invasive fungal infections, in particular those affecting immunocompromised patients, still present high morbidity and mortality rates (Wenzel and Edmond, 2001) often due to late and inaccurate diagnosis. These limitations in diagnosis often hamper the application of proper and timely antifungal therapy. In fact, several studies have pointed out that early and accurate diagnosis, together with the subsequent early initiation of therapy improves the outcome of the disease (Barnes, 2008). Additionally, the use of antifungal prophylactic strategies in patients most at risk of these infections is resulting in the emergence of fungal pathogens with an increased resistance to the drugs commonly available; newly emerging fungal species include not only species of *Candida* other than *C. albicans*, but also *Aspergillus* spp. and other filamentous fungi. In that sense, optimal patient management will require not only the development of new diagnosis strategies able to cope with the current limitations of the available methodologies, but also the identification of high-risk groups in which the prevalence of fungal infection is known to be increased.

This dissertation was developed within the abovementioned problematic, envisaging that necessary investigations in this field including the development of accurate and rapid diagnostic methods, and that a step forward could be given in risk stratification with the identification of human genetic susceptibility markers allowing identification of patients most at risk.

Accurate and timely diagnosis of fungal infections remains a problematic issue regarding immunocompromised patients, in whom signs and symptoms are non-specific and often develop late in the course of infection. Even though *C. albicans* is still the most common and clinically relevant fungal pathogen, other species, often resistant to the currently available antifungal agents, have been recently emerging. These observations led both researchers and clinicians to an intensive development of alternative diagnostic methods in recent years allowing identification

of *Candida* to the species level. In this sense, we developed a multiplex PCR-based method with a test matrix able to identify eight of the most clinically relevant *Candida* species, namely *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *C. dubliniensis* (Chapter 2). The designed diagnostic method allows the identification of yeasts that have non-standard morphologic, cultural and biochemical characteristics with high specificity, devoid of interference by other microorganisms that commonly co-infect immunocompromised patients. Additionally, under laboratory conditions, using artificially seeded blood, the method revealed a high sensitivity, allowing the identification of approximately 2 live/intact *Candida* cells per ml of blood. This attribute, together with the reduced time required for identification to a minimum of 5 hours, will allow clinicians to detect candidaemia in its early stages of development, where few circulating *Candida* cells are present. Altogether, these features point to a highly advantageous application in the detection and identification of *Candida* species in both clinical diagnosis and epidemiological studies, although validation in clinical settings is still required.

As referred above, and despite common immunosuppression, not all patients develop fungal disease, thus suggesting that in addition to characteristic traits of the fungi involved, individual genetic variations may contribute to their onset. Recently, considerable attention has been given to variations in genes of the innate immune response such as Toll-like receptors (TLRs) and their potential association with susceptibility to infectious disease (Schroder and Schumann, 2005). In the same way, these variations could underlie an increased predisposition to infections of fungal nature, both invasive as well as non-invasive. Recently, extensive research coverage has been given to association studies between the presence of single nucleotide polymorphisms (SNPs) and differences in susceptibility to diseases of diverse aetiologies, both infectious and non-infectious. The fact that most of the methods currently available to assess polymorphisms are either time-consuming or laborious, requiring expensive equipment, led us to adapt a simple and rapid method, allowing genotype determination in a single PCR reaction (Chapter 3). Accordingly, genotyping of TLR polymorphisms performed in the association studies depicted in Chapters 4, 5 and 6 was carried out using this methodology. Moreover, the work developed in Chapter 3 illustrates, to the best of our knowledge, the first characterization of the general Portuguese population regarding polymorphisms in TLRs. The allele frequencies of the different studied polymorphisms did not significantly differ from those described in the literature for other Caucasian populations (Vogel et al., 2005). The results presented can be of further use

in association studies for different disorders in the Portuguese population. Also, taking into account the role of these polymorphisms and susceptibility to infectious disease, the data presented provides valuable information that can be potentially used to stratify patients most at risk regarding infection as highlighted in Chapters 4, 5 and 6.

As previously stated, the role of TLRs in the recognition of pathogenic fungi and the initiation of the inflammatory response led us to raise the hypothesis that individuals carrying polymorphisms in TLR genes could display differential levels of protection against fungal infection. Although in the beginning of this dissertation project, we intended to study how the genetic background, particularly regarding TLR genes, could contribute to both infections with *Candida* and *Aspergillus* species, mostly due to sample limitations, the developed work focused primarily in susceptibility to different forms of aspergillosis. TLR4 has always displayed an intriguing role in the recognition and initiation of the inflammatory response to *Aspergillus*. Experimental evidence indicates TLR4 as one of the most important innate immune pathways in response to the fungus (Bellocchio et al., 2004a; Braedel et al., 2004; Chignard et al., 2007; Mambula et al., 2002; Netea et al., 2003). Accordingly, we found that a *TLR4* polymorphism - Asp299Gly - was highly associated with the development of a non-invasive form of pulmonary aspergillosis, chronic cavitary pulmonary aspergillosis (CCPA) (Chapter 4). Furthermore, we found a positive association between the same SNP and fungal colonization but not susceptibility to fungal infection in haematopoietic stem cell transplanted (HSCT) patients (Chapter 5). These results suggest that the failure to recognize the fungus due to a defective TLR4 extracellular domain may be predisposing to fungal colonization, in the same way it occurred for CCPA. However, this effect may be compensated by the lack of an enhanced inflammatory response to the fungus due to impairment in the production of proinflammatory cytokines, thus contributing to fungal infection resistance. Despite a comprehensive knowledge on *TLR4* polymorphisms and susceptibility to infectious and non-infectious diseases, their role in the standard performance of the immune system is still obscure. Thus, since Asp299Gly appears to be essential in the outcome of the host-*Aspergillus* interaction, functional studies regarding the impact of this polymorphism on TLR signalling pathways are imperative. For such, the evaluation of the cytokine profile triggered by *Aspergillus* species in human cell lines carrying Asp299Gly could shed light in the mechanism underlying the genetic associations herein presented. In addition, the modulation of gene expression of other TLRs, namely *TLR2*, in association with the different *TLR4* genotypes, could give further insights on the balance of the Th1/Th2 types of response

regarding the relationship between local colonization and disseminated infection. Overall, functional studies regarding the impact of this polymorphism on TLR signalling pathways will allow the design of new targeted antifungal therapeutics taking into consideration the *TLR4* genotype of each individual.

On the other hand, the *TLR9* polymorphism T-1237C, previously linked to an increased predisposition to several diseases of chronic inflammatory nature, was shown to be associated with susceptibility to both ABPA (Chapter 4) and viral infection (Chapter 5). Thus, taking into account the critical role of TLR9 in viral sensing, assuring rapid antiviral responses, as well as in the control of allergy to fungi, a putative disease mechanism involving this polymorphism would be an exuberant inflammatory response due to increased *TLR9* expression levels and concomitant receptor stimulation. Accordingly, a shared disease mechanism was also proposed as a risk factor for developing non-Hodgkin lymphoma (NHL) (Chapter 6). The presence of the T-1237C polymorphism was shown to introduce a new regulatory sequence in the *TLR9* promoter, *trans*-activated by an IL-6-dependent transcription factor, ultimately resulting in increased *TLR9* expression. This effect, together with persistent receptor activation, was shown to lead to increased B lymphocyte proliferation and augmented IL-6 production by these cells harbouring the mutant allele, ensuing cell-specific uncontrolled proliferation (Chapter 6). These abnormally high proliferation indexes can make cells more prone to the acquisition of specific transforming mutations leading to the development of NHL, such as those occurring in the *bcl-2* or *c-myc* genes. Furthermore, the use of blocking agents of the pathways involved in this disease mechanism, such as neutralizing anti-IL-6 antibodies or TLR9 antagonists that limit the outcome of its signal transduction pathway, was shown to inhibit the pronounced effects of the polymorphism on gene expression, proliferation and IL-6 production. The proposed susceptibility model, as well as the suggested targeted therapeutics, namely the use of anti-IL6 antibodies or TLR9 antagonists, requires future confirmation in NHL patients. In addition, the recent use of CpG agonists in the treatment of cancer and autoimmune diseases should be seen with moderate distrust, as individual variations affecting the outcome of the TLR9 signalling pathway suggest the requirement of individual tailored CpG-based treatments depending on the *TLR9* genotype.

The results and considerations presented throughout this dissertation reinforce the requirement for changes in the management of fungal infections. First, at the fungus level, with the development of better diagnostic procedures ensuing proper and timely establishment of

antifungal therapy that can potentially contribute to a reduction in the high mortality rates associated to these diseases. Second, at the host level, with the understanding of the impact of individual genetic variations in the outcome of the host-fungi interaction that may allow stratification of patients most at risk of infection and the use of alternative therapeutic strategies. These conclusions reinforce the need for future studies elucidating the effect of TLR polymorphisms both at molecular and cellular level. More specifically, the characterization of the balance between Th1/Th2 cytokine profiles resulting from the stimulation of defective TLRs should be elucidated in the future, allowing the clarification of the impact of these polymorphisms in immune cell function and activation of the adaptive immune response in *in vitro* models of infectious disease. This will allow the description of functional models depicting the correlation between the discovered functional alterations and susceptibility to infectious diseases, thus identifying therapeutic targets and/or strategies able to exert control over TLR signalling pathways and thus minimize the defects in cell function underlined by the polymorphisms.

In summary, the information presented in this dissertation further highlights the importance of TLR polymorphisms in susceptibility/resistance to fungal infections, both in its invasive and non-invasive forms. Additionally, the shared mechanisms affected by TLR polymorphisms may predispose to diseases of variable aetiology, as was shown for T-1237C and NHL. Altogether, the information regarding individual predisposing variants may potentially be used in the future, allowing the formulation of individually tailored therapeutic approaches.

CHAPTER 8

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