Diagnosis of Aspergillosis: Role of Proteomics

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The expansion of the antifungal armamentarium and the implementation of imaging techniques and new nonculture-based fungal diagnostics (NCBFDs) have improved the survival of patients with invasive aspergillosis (IA). However, mortality rates still remain high, possibly influenced by several pitfalls, affecting NCBFDs and reducing the window of opportunity for earlier treatment. A large body of in vitro and in vivo studies has demonstrated that several fungal proteic components are strongly immunogenic, and both the adaptive immunity and the innate branch are heavily involved in the recognition and clearance of fungal pathogens, resulting, on occasion, in a useful tool for the treatment of IA. By evaluating these studies, this review considers the possibility of exploiting either components of the innate or adaptive immunity to support the rapid and early diagnosis of IA.

Introduction

The new antifungal compounds and the improvement in fungal diagnostics have increased the survival of patients affected by invasive aspergillosis (IA) [1•,2•]. Voriconazole, liposomal amphotericin B, and caspofungin have been reported to allow response rates ranging from 33% to 53% in patients with proven or probable disease [3••,4,5]. These results have been obtained with the aid of nonculture-based fungal diagnostics (NCBFDs), namely the detection of the fungal antigens galactomannan and β-D-glucan, and the detection of fungal DNA by polymerase chain reaction (PCR) techniques. These new tools have been demonstrated as valuable supports for the diagnosis of IA in several clinical studies, allowing earlier therapeutic intervention [6•,7,8•]. Galactomannan antigenemia and β-D-glucan are already widely used and have shown an elevated negative predictive value (ranging from > 95% to 100%), suggesting that both assays are extremely effective in ruling out the diagnosis of IA [9•,10]. These results have recently led the European Organization for Research and Treatment of Cancer Mycoses Study Group (EORTC/MSG) to introduce both tests as a microbiological criterion to define probable IA in patients with the appropriate radiologic findings [11••]. Moreover, a recent meta-analysis reported that PCR shows a sensitivity and specificity of 88% and 75%, and 75% and 87%, when a single or two positive consecutive samples are considered, respectively [12]. Thus, it has been suggested that a single PCR-negative result may be sufficient to exclude, while two positive tests are required to confirm, the diagnosis of IA [12].

However, the mortality rates, still ranging from 13% to 70% in hematologic centers, and the clinical costs, being one third higher in patients with proven or probable IA than in patients without IA, stem in part from the difficulties of NCBFDs to allow a timely diagnosis on several occasions [2•,13•]. The variability in performance represents the main shortcoming with NCBFDs, due to heterogeneities between studies, the possibility of false-positive and false-negative results with the concomitant use of parenteral solutions, semisynthetic β-lactam antibiotics, and antifungal prophylaxis, and the lack of standardization. The latter is a major factor hindering the routine and broadened use of PCR in the diagnosis of IA [8•,9•].

Thus, the possibility has been raised to explore new diagnostic tools that might refer to host factors, such as the immune responses elicited by pathogens, rather than to fungal cell components, and that might be used in conjunction with modern imaging techniques and NCBFDs, in order to possibly overcome their pitfalls.

The Role of Adaptive Immunity and Pentraxins in Mouse Models of Invasive Fungal Infections

Several studies have addressed the role of the antigen-specific immune responses and of the preferential stimulation of CD4+ T helper (Th) cell subsets in the mouse model of

IA. In the seminal study by Cenci et al. [14], mice splenocytes, obtained after injection of either lethal or sublethal inoculums of *Aspergillus fumigatus*, were assessed at 2 and 7 days, respectively, for the production of interferon (IFN)-γ, interleukin (IL)-4, and IL-10. Mice surviving IA produced higher IFN-γ and lower IL-4 levels than mice lethally injected and showed significant decreased production of IL-10 at 7 days. Moreover, the evaluation of IFN-γ, IL-4, and IL-10 gene expression to correlate the above-mentioned findings with the presence of CD4+ Th1 and Th2 cell subsets in vivo demonstrated that in mice surviving IA, IFN-γ and IL-10 RNA messengers were always present during the course of the disease, whereas IL-4 RNA messenger strongly decreased late in the infection. By showing a differentially regulated cytokine production, this study demonstrated that the predominance of IFN-γ–producing T cells was protective against IA, whereas the predominance of IL-4– and IL-10–producing T cells was permissive to IA [14].

Further insights to the role of IL-10–producing T cells in IA have been derived from Del Sero et al. [15]. These authors showed that IL-10–deficient (IL-$10^{-/-}$) mice demonstrated extreme resistance to invasive fungal infections (IFIs), including IA, in virtue of the elevated amount of IFN-γ. Nevertheless, IL-10–sufficient mice maintained the ability to mount a protective Th1 response in the course of IFI, by producing adequate amounts of IFN-γ. Of note, a few IL-$10^{-/-}$ mice presented a dismal outcome after the resolution of the IFI, due to an excessive inflammatory response [15].

In line with these and subsequent studies, it has been suggested that, in the course of IFI, the production of IL-10 may play a double role, namely a permissive role to a fungal infection, allowing the fungi to persist, but also a regulatory role in the production of IFN-γ, possibly allowing IFN-γ to be effective against fungi but not deleterious for the hosts, avoiding an excessive inflammatory response [15,16].

A protective Th1 response also seems to be strictly influenced by the presence of a protein involved in the innate response to pathogens, namely the long pentraxin 3 (Ptx3), which is thought to play a nonredundant role in immunity. Ptx3-null mice are extremely susceptible to IA [17]. They show either a defective recognition of fungal conidia or an unbalanced cytokine profile skewed toward a Th2 response, demonstrated by high levels of pulmonary IL-4 and low levels of either IFN-γ or IL-12. Consistent with this, the administration of Ptx3 was protective in mice undergoing bone marrow transplantation, and was associated with an accelerated recovery of lung phagocytes and Th1 lymphocytes, again demonstrating its nonredundant role [17].

Recently, it has been reported that IL-10–deficient mice may show attenuated pulmonary *A. fumigatus*–specific Th responses with aberrant intestine localization of fungus-specific CD4+ T cells [18]. The de-localization may be reversed by antibiotics without any antifungal activity. Although obtained in immunocompetent mice, focusing only on *A. fumigatus*–specific CD4+ T-cell responses and apparently in contrast with previous observations, the study points to a possible further function of IL-10 in the course of IA, potentially useful either in maintaining the intestinal mucosal integrity or in controlling the recirculation of specific T cells to the site of infection [18].

In addition, the crucial role of T cells in the defense of mice against fungi has been supported by the demonstration that CD4+ Th1 response may be elicited in immunocompetent mice, after the inhalation of a crude *A. fumigatus* filtrate, and may be adoptively transferred to naïve mice, with a protective result [19]. Of note, no signs of activation of protective immunity at distant sites were detected in mice infected intragastrically. On the contrary, the administration of the recombinant protein Asp f2, encoding for a major allergen of the fungus, did not result in a transferable response, because no recall immunity could be elicited, but, conversely, resulted in the persistence of the pathogen by the stimulation of high levels of inhibitory IL-4 and IL-10 [19].

However, these findings, although suggesting that fungal antigens could be useful candidate vaccines against IA, raise several still unresolved issues about the possibility that different sites of infection, different times of fungal exposure or clearance, and different *Aspergillus* antigens may strongly influence the occurrence of either a protective or permissive immunity in the course of IA.

Finally, it has recently been reported that the administration of an *A. fumigatus* extract in ovalbumin-specific T-cell receptor transgenic mice may elicit not only specific CD4+, but also CD8+ immune responses that may be adoptively transferred to syngeneic naïve recipients [20]. Remarkably, the increased proliferation of the specific CD8+ T cells was strongly accompanied by a large differentiation into IFN-γ–producing cytotoxic T lymphocytes, whereas the specific CD4+ cell activation and expansion were devoid of detectable effector differentiation [20].

However, the observation that fungal gliotoxin may suppress the perforin-mediated cytolytic activity of CD8+ cell clones in vitro still questions the efficacy of this T-cell subset in IA [21].

Moreover, a role of IL-17–producing CD4+ Th cells (Th17) in IFI has been demonstrated by showing that Th17 pathway expression correlates directly with defective pathogen clearance and the failure to resolve inflammation as well as to initiate protective responses to *Aspergillus* [22•].

The Role of Adaptive Immunity and Pentraxins in Human Fungal Infections

The studies reporting that IA may also be detected in non-neutropenic patients, namely those with advanced AIDS, preterm neonates, solid organ transplant (SOT) and stem cell transplant (SCT) patients, and in apparently immunocompetent patients (ie, those with chronic obstructive

lung disease, liver cirrhosis, and diabetes, mainly residing in intensive care units), seem to suggest that defects of adaptive immunity may represent a major predisposing factor to IA in humans as well [23,24,25•,26•].

Roilides et al. [27–29] measured the levels of serum IL-10 in seven non-neutropenic immunocompromised patients with IA, starting from the in vitro observations that the addition of human IL-10 decreased the fungicidal activities of either monocytes or polymorphonuclear cells when cultured with fungal hyphae or conidia. They distinguished two clinical patterns: the former, observed in those with persisting low serum IL-10 levels, was associated with either stable or partially responsive disease; the latter, observed in those with increasing serum IL-10 values, was associated with progressive disease [29]. The authors concluded that serum IL-10 levels could be evaluated as a marker of disease progression and that the IL-10 blockade could have been exploited as a possible therapeutic option [29]. However, whether these results could have really represented a Th2 immune response, triggered and favored by *Aspergillus*, remained uncertain.

Hebart et al. [30] performed the first study exploring the role of T-cell–mediated immunity in hematologic patients with IA. Peripheral blood mononuclear cells (PBMCs) from 16 healthy volunteers were cultured for 5 days with antigens derived from *Aspergillus* hyphae, conidia, and enzymes, showing a significant lymphoproliferative response (stimulation index 3 or higher) in 14 of 16, seven of eight, and eight of 11 healthy individuals, respectively. Furthermore, a cytokine secretion assay revealed that the culture supernatants of PBMCs from 13 of 17 and from 11 of 17 healthy subjects stimulated with hyphal and enzymatic antigen, respectively, contained IFN-γ concentrations at least twofold higher than IL-10 concentrations, suggesting that the *Aspergillus*-specific T-cell response was polarized to the production of IFN-γ in healthy volunteers [30].

The same analyses were performed on PBMCs from allogeneic SCT (alloSCT) patients, affected by IA, namely in five proven, three probable, and 12 possible IA cases. Of note, 14 of 18 evaluable patients presented a positive lymphoproliferative response. Interestingly, the release of IFN-γ and IL-10 in culture supernatants demonstrated a higher IFN-γ/IL-10 ratio in patients with favorable outcome of IA, and a lower IFN-γ/IL-10 ratio in patients with progressive or stable disease, in spite of the antifungal treatment [30]. The evaluation of lymphoproliferative responses to *Aspergillus* antigens resulted positive in four of 18 alloSCT patients not affected with IA, with a low stimulation index and a low IFN-γ/IL-10 ratio, possibly indicating a Th2 polarized immune response, potentially contributing to the prolonged risk of IA in this patient group [30].

These findings suggest that protective immunity against IA correlates with Th1 responses, whereas a Th2 cytokine pattern may be permissive to IA not only in mice but also in humans.

A few years later, it was reported that anti-*Aspergillus* T-cell clones, producing IFN-γ when stimulated with *Aspergillus* antigens in vitro, may be adoptively transferred from donors to recipients of haploidentical SCT [31]. Interestingly, after immunotherapy, T-cell clones from the recipients showed a "protective" higher IFN-γ/low IL-10 ratio, similar to what was observed in donors. On the contrary, in control patients not undergoing the adoptive transfer, T-cell clones recovering spontaneously displayed a "nonprotective" lower IFN-γ/high IL-10 ratio when stimulated with antigens from the corresponding pathogen [31].

Moreover, in nine of 10 patients with pneumonia and positive galactomannan antigenemia, the adoptive therapy resulted in disease resolution and in either the reduction or the normalization of galactomannan values, without causing episodes of graft-versus-host disease [31].

Although the treated patients had probable but not proven IA, and the specificity of the T-cell clones for *Aspergillus* was not formally demonstrated by excluding that they could also be functionally active against other filamentous fungi, the authors documented the antifungal efficacy of T-cell therapy for the first time [31].

More recently, it has been demonstrated that anti-*Aspergillus*–specific T cells may be generated, enumerated, and functionally characterized in healthy individuals and alloSCT patients [32••,33•,34].

In their first study, Beck et al. [32••] obtained anti-*Aspergillus*–specific T cells from five healthy volunteers. The cells produced IFN-γ and IL-2, but not IL-4 and IL-10, when stimulated with antigen extracts of *A. fumigatus*, indicating that a Th1 response was induced. These Th1 cells also responded upon stimulation with *Aspergillus flavus*, *Aspergillus niger*, and *Penicillium chrysogenum* antigens, but not with *Aspergillus alternaria* or *Candida albicans*, and showed the ability to induce hyphal damage, either directly or when associated with polymorphonuclear and antigen-presenting cells. Furthermore, the anti-*Aspergillus*–specific T cells were not affected in their functionality by cryopreservation up to 6 months and could be expanded after restimulation with appropriate antigens. The authors have raised the possibility of generating anti-*Aspergillus*–specific T cells on a clinical scale, with a rapid and simple method within 13 days [32••].

In further studies, the same authors analyzed PBMCs from 23 healthy donors and from 15 patients undergoing alloSCT by means of flow cytometry and intracellular cytokine staining. The healthy subjects had between one and 12 anti-*Aspergillus*–specific T cells per μL, exhibiting a memory CD4+ T-cell phenotype ($CD3^+$, $CD4^+$, $CD45RO^+$) [33•,34]. On the contrary, in alloSCT patients, anti-*Aspergillus*–specific T cells were not detectable within the first 4 months after transplantation or were significantly lower than in healthy individuals up to 1 year post-transplant [33•,34].

Recently, Zhu et al. [35] reported the feasibility of generating not only specific CD4+, but also specific CD8+ T-cell lines against the *A. fumigatus* antigen f16, by using

co-culture of dendritic cells (DCs) and lymphoblastoid cell lines, pulsed with a pool of pentadecapeptides spanning completely the coding region of f16, with PBMCs from healthy subjects. Notably, such a priming seems to produce *A. fumigatus*–specific T cells, earlier than using DCs alone and with more potent cytotoxic activity, in virtue of a higher frequency of IFN-γ–producing T cells [35].

Although conducted on a small series of patients and raising several issues about the optimal antigen(s) to be used for reproducibly generating the anti-*Aspergillus*–specific T cells and about the culture and stimulation techniques to reduce the long-term in vitro period allowing enrichment of pathogen-specific T cells, these studies seem to demonstrate that there is a direct correlation between the frequency of anti-*Aspergillus*–specific T cells and the risk of developing IA, similarly to what was observed for the absolute number of neutrophils. Nonetheless, it remains to be defined which is the protective threshold of anti-*Aspergillus*–specific T cells, which patient populations may simply benefit from antifungal prophylaxis or ultimately need the transfer of anti-*Aspergillus*–specific T cells, and whether the immunotherapeutic strategies could even involve autologous T cells.

Finally, only one study has addressed the function of Ptx3 in humans, by showing that the protein was significantly increased in plasma of 10 hematologic patients with IA and was highly expressed in alveolar macrophages and in a few circulating mononuclear cells. Unfortunately, the authors did not provide information on the clinical outcome nor on the evaluation of the adaptive immune response in these patients [17].

Specific T Cells for the Diagnosis of Infectious Diseases: Lessons From Viral Infections and Tuberculosis

Data from the organ transplantation setting have shown that the higher the risk for viral infections and viral-associated complications, the lower the antiviral-specific cellular immunity [36]. Impaired immune reconstitution of Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpes virus (HHV)-8, and polyomavirus BK (BKV)–specific T cells after alloSCT or SOT contributes to an increased susceptibility to viral replication and potentially life-threatening viral diseases by altering the equilibrium between host defense and the pathogens [36–41]. Thus, by allowing the identification of patients with defective or absent specific T-cell immunity, quantification of EBV-, CMV-, HHV-8–, and BKV-specific T cells has been adopted as a diagnostic tool to support clinical decision making on antiviral and/or immunosuppressive therapy regimens. The detection of antiviral immune responses may complement the routine viral load monitoring, to better identify the transition from the infection to disease [36–41].

Moreover, in the past few years recent advances in the basic science of tuberculosis research have overcome the limitations represented by traditional tools for the diagnosis and prevention of tuberculosis infection (TBI) [42]. The studies in mycobacterial genomics have led to the identification of two proteins encoded by the region of difference-1 of *Mycobacterium tuberculosis*, namely early secretory antigenic target-6 and culture filtrate protein-10, which resulted in strong targets of Th1 cells in patients with *M. tuberculosis* infection. Thus, measuring the IFN-γ–producing antigen-specific T-cell responses by means of either an enzyme-linked immunospot (ELISPOT) assay or a whole-blood enzyme-linked immunosorbent assay has demonstrated a higher sensitivity (ranging from 83%–97%), compared with that of the traditional tuberculin skin test, in the diagnosis of either active or latent tuberculosis [42,43]. The high diagnostic accuracy of both blood tests represents a step forward in the program of tuberculosis elimination, by allowing better recognition of patients inappropriately treated and more accurate targeting of individuals with latent TBI and at higher risk for progression to overt infection [42,43].

The Study of the Immune Response for the Diagnosis of IA

Only two studies have addressed the possible value of monitoring Ptx3 serum levels in the diagnosis of IA [44,45].

In the first study, al-Ramadi et al. [44] reported that five patients (two with proven and three with probable IA) showed normal or reduced levels of Ptx3 during IA, suggesting a possible role of alveolar macrophages in reducing the serum levels of the protein. However, these discouraging results were probably related to a low sampling frequency, because only one sample for each patient was measured concomitantly with the infectious process [44].

More recently, Biagi et al. [45] reported that the serial monitoring of Ptx3 plasma levels (once a week for at least 6 weeks from the diagnosis of infection) may be used as a supplementary tool to strengthen the diagnosis of IA, by showing that in 10 immunocompromised children with pulmonary IA (seven possible and three probable IA according to EORTC/MSG criteria) the levels of the protein were higher than in children without infections and that the Ptx3 kinetics correlated well with clinical outcome [45].

Furthermore, a group from the University of Modena and Reggio Emilia recently developed and patented an ELISPOT assay to track individual *Aspergillus*-specific T cells, either producing IFN-γ (IFN-γ–Th1) or IL-10 (IL-10–Th2) [46•,47,48]. The assay is based on the principle that T cells from individuals with *Aspergillus* species infection may become sensitized to *Aspergillus* species antigens in vivo and may possibly be detected in the peripheral blood. In the overnight ELISPOT assay, when the T cells re-encounter these antigens ex vivo, they may release cytokine, either IFN-γ or IL-10, according to the cytokine antibodies used on the pre-coated wells. After brief incubation, each antigen-specific T cell gives rise to a dark spot, which represents the footprint of a single *Aspergillus*-specific T cell. The evaluation of the assay

consists of the enumeration of spots through a magnifying lens or automated reader. The antigens used to elicit such a recall-specific immune response are represented by *A. fumigatus* proteic extract deriving from watered hyphae and conidia killed by heating.

Thus far, 12 hematologic patients have been reported: five affected by proven IA and seven considered control patients, because they were affected by infectious processes of known etiology other than IA, on the basis of cultural and/or histologic examinations. All but two patients presented pulmonary infiltrates diagnosed by high-resolution CT, whereas the remaining two showed liver and spleen lesions detected on abdominal ultrasonography and CT scans, respectively [46•,47,48].

ELISPOT was performed at different time points during the course of the disease in proven IA patients (median, four samples per patient), and during radiological diagnosis of the pulmonary infiltrates in the control patients.

The assay provided the proof for proven IA, resulting positive for the presence of *Aspergillus*-specific T cells, either IFN-γ–Th1 or IL-10–Th2, at any time, in all but one sample. This latter sample was collected at the time of acute myelogenous leukemia overt relapse and had an undetermined result for the presence of evaluable specific responses. Moreover, the assay represented the only proof of IA in two of five patients, in whom the diagnosis had been reached only after surgical intervention, being all the other available clues, including galactomannan, were repeatedly negative [46•,47,48].

The ELISPOT assay was negative for both *Aspergillus*-specific IFN-γ–Th1 and IL-10–Th2 in the patients affected by pulmonary diseases other than IA. Of note, all of the patients were receiving antifungal prophylaxis with itraconazole (400–600 mg/day orally), and this did not seem to influence the assay's performance [46•,47,48].

Furthermore, the ELISPOT assay seemed to provide the description of the kinetics of *Aspergillus*-specific T-cell responses in vivo in humans during the course of IA, by showing the sequential skewing from a permissive immunity polarized to IL-10–Th2 at the onset of IA to a protective immune response polarized to IFN-γ–Th1 at the complete resolution of the disease, reminiscent of what was reported in mouse models of IA and in cultures of PBMCs with *Aspergillus* antigens from IA patients [46•,47,48].

Conclusions

The wider use of NCBFDs has sensibly increased the survival of patients affected by IA, often allowing an early diagnosis. At some institutions, the high diagnostic accuracy of such methods has also allowed a preemptive therapeutic approach, based on the concept successfully applied to viral infections in SOT and SCT patients [49,50•]. Nonetheless, PCR still needs validation and standardization, and several clinically important issues remain to be addressed about the other NCBFDs, especially regarding the frequency of sampling, the confirmation of positive results, the management of false-positive or false-negative results, and the performance of the tests in established categories of patients, including pediatric and nonhematologic patients, or upon antifungal prophylaxis. Once more, the model of viral infections could inspire clinicians handling IA, who could usefully consider the possibility to improve the diagnosis of IA, through the study of pathogen-specific immune responses. A few reports have already suggested that the quantification or the enumeration of innate and adaptive immunity components may be useful in the diagnosis of IA. In particular, the ELISPOT assay, by assessing *Aspergillus*-specific T cells, looks promising, and also because it shifts the attention to host factors, which are more specific and less likely to be influenced by confounding elements.

However, we eagerly await confirmations of the clinical value of quantification of Ptx3 or detection of *Aspergillus*-specific T cells in case-control and prospective studies.

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Disclosure

No potential conflicts of interest relevant to this article were reported.

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