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# Manipulating immunity against *Aspergillus fumigatus*

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Efficient response to *Aspergillus fumigatus* requires different mechanisms. Polymorphonuclear neutrophils (PMNs) are the predominant immune cells in the acute stage of most fungal infections and play a crucial role in determining the type of pathology associated with fungal infections in different clinical settings. Dendritic cells (DC) are able to decode the fungus-associated information and translate it into different T helper (Th) and regulatory (Treg) cell responses. Functionally distinct Treg cells are activated after exposure to *Aspergillus* conidia. Early in infection, inflammation/Th1 reactivity is controlled by Treg cells suppressing PMNs and the immunogenic program of DC. The levels of IFN-γ produced in this phase set the subsequent adaptive stage by conditioning the indoleamine 2, 3-dioxygenase (IDO)-dependent tolerogenic program of DC and the subsequent activation of tolerogenic Treg cells, which inhibit Th2 cells and prevent allergy to the fungus. Knowledge of the immunopathogenesis of *Aspergillus* infections may pave the way to promising strategies for immunotherapy.

**Keywords** *Aspergillus fumigatus*, inflammation, allergy, regulatory T cells, T helper cells, indoleamine 2, 3-dioxygenase, dendritic cells, polymorphonuclear neutrophils

## Introduction

*Aspergillus fumigatus*, a thermotolerant saprophyte, is associated with a wide spectrum of diseases in humans that includes saprophytic colonization of pre-existing cavities (aspergilloma), allergic asthma, allergic bronchopulmonary aspergillosis occurring as a complication of bronchial asthma or cystic fibrosis, and invasive aspergillosis in immunocompromised patients. Immunocompetent and non-atopic subjects are relatively resistant to *A. fumigatus* diseases and disease occurs in the setting of host damage [1,2]. Most of the inhaled conidia is eliminated by exclusion mechanisms, which include physical barriers, such as mucus, and cilia as well as a variety of mediators of the collectin family, such as lung surfactant proteins SP-A, SP-D, mannose-binding lectins (MBL) and pentraxin 3 (PTX3), with anti-microbial and immunomodulatory properties [3–5]. Patients with single nucleotide polymorphisms in SP-A2 and MBL genes showed significant associations with *Aspergillus* infection and allergy [5]. Many aspects of the antimicrobial host response are orchestrated by a complex network of cytokines and their receptors [6]. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 have been shown to be required for initiation of the innate response to the fungus [6,7]. Several recruitment chemokines play critical roles in mediating influx of specific leukocytes to the site of infection in experimental pulmonary aspergillosis [6]. Of activating cytokines, those associated with the Th1 phenotype, including IL-12, IL-18, and interferon (IFN)-γ, are critical for protective responses to the infection. Conversely, the Th2 cytokine IL-4 contribute to progression of infection [6,8].

Although epithelial and endothelial cells may internalize conidia [1], effector mechanisms of the innate immune system have long been recognized as major host defenses against invasive aspergillosis [1,9]. Resident alveolar macrophages ingest inhaled conidia very rapidly, destroy them intracellularly through oxidative mechanisms and prevent germination to hyphae, the

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invasive form of the fungus. PMNs, through oxygen and non oxygen-dependent mechanisms, attack hyphae germinating from conidia that escape macrophage surveillance. PMNs are the predominant immune cells in the acute stage of the infection and are essential in initiation and execution of the acute inflammatory response and subsequent resolution of the infection. If the above lines of host cellular defences are suppressed (e.g., by corticosteroids) or absent (e.g., as a result of neutropenia), the fungus can germinate into hyphae and invade the lung parenchyma and blood vessels, producing tissue infarction, hemorrhagic necrosis, and death. In some, but not all, patients who remain persistently and profoundly immunocompromised, *A. fumigatus* can disseminate to distal sites including the brain, kidney, liver, and skin. The pathogenic determinants responsible for distal seeding of *A. fumigatus* to target organs are unknown.

This review highlights pathways of immuno-resistance to the fungus that have proven to be amenable for immunotherapeutics in murine experimental models of pulmonary invasive aspergillosis.

## Toll-like receptor on PMNs: a division of labor

PMNs are essential in initiation and execution of the acute inflammatory response and subsequent resolution of fungal infection. PMNs, however, may act as double-edged swords, as the excessive release of oxidants and proteases may be responsible for injury to organs and fungal sepsis. Recognition of *Aspergillus* and activation of PMNs occurred through the involvement of distinct members of the Toll-like receptor (TLR) family, each likely activating specialized antifungal effector functions. By affecting the balance between fungicidal oxidative and nonoxidative mechanisms, pro- and anti-inflammatory cytokine production, and apoptosis vs necrosis, the different TLRs ultimately impacted on the quality of microbicidal activity and inflammatory pathology. Signalling through TLR2 promoted the fungicidal activity of PMNs through oxidative pathways involving extracellular release of gelatinases and proinflammatory cytokines while TLR4 favoured the oxidative pathways through the participation of azurophil, myeloperoxidase-positive, granules and IL-10. This translated *in vivo* in the occurrence of different patterns of fungal clearance and inflammatory pathology. The ability of selected individual TLRs to restore antifungal functions in defective PMNs suggests that the coordinated outputs of activation of multiple TLRs may contribute to PMN function in aspergillosis (Fig. 1). Although TLRs signalling may result in contrasting outputs in different types of effector cells [10], including in response to *Aspergillus* [11–15], it is reasonable to believe that manipulation of TLRs by selective agonists might provoke divergent sequences and magnitudes of functional responses, so that diverse outcomes ultimately may transpire. Although much remained to be learned as to whether TLRs ligands exert their effects directly through TLRs signalling or through other indirect mechanisms, the ability of selected TLRs ligands to restore the defective antifungal activity of PMNs from transplanted patients or to antagonize the steroid-induced immunosuppression [16] suggests that the coordinated outputs of activation of multiple TLRs may govern the function of PMNs in aspergillosis. It is conceivable that a better understanding of the TLR biology on PMNs may provide rationale bases for granulocyte transfusions in neutropenic patients with a life-threatening *Aspergillus* infection.

## Dendritic cells: a concerted action of distinct subsets

*Aspergillus* proved to be useful pathogen model to dissect events occurring at the fungus/DC interface. DC are uniquely able to decode the fungus-associated information and translate it in qualitatively different Th immune responses, *in vitro* and *in vivo* [17–21]. The DC system comprises a network of different subpopulations [18–20]. DC subsets differ in their phenotype, micro environmental localization, migration potential, PRR expression, responsiveness to microbes, and their

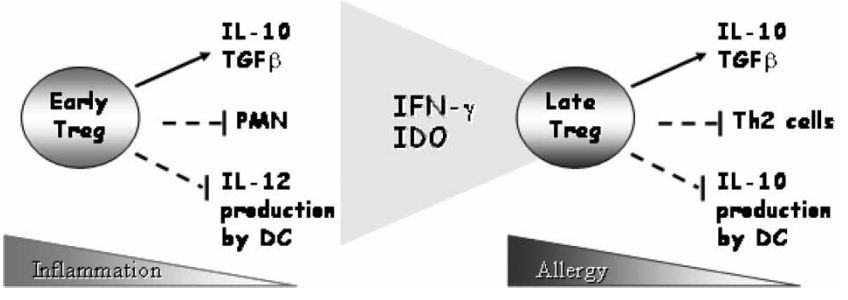


**Fig. 1** Schematic representation of possible correlations between pathogen control and inflammatory response on polymorphonuclear neutrophils exposed to *Aspergillus fumigatus* in different TLR context. See text for details.

capacity to induce and regulate distinct arms of the innate and adaptive immune systems [22]. By using distinct pattern recognition receptors, including TLRs, human and murine conventional (cDC) and plasmacytoid (pDC) DC were able to finely discriminate between conidia and hyphae of *Aspergillus* in terms of induction of adaptive Th responses [11,18,19,22]. The fungus has exploited common pathways for entry into DC which include a lectin-like pathway for the unicellular form and opsono-dependent pathways for the filamentous form. Recognition and internalization of unopsonized conidia occurred through the engagement of mannose receptors (MRs) of galactomannan specificity, DC-SIGN and, partly, complement receptor 3 (CR3). In contrast, entry of hyphae occurred by a more conventional, zipper-type phagocytosis and involved the cooperative action of FcγR II and III and CR3. Actually, the sugar specificity of MRs involved in the entry of one or multiple *Aspergillus* conidia turned out to be different, as the entry of multiple conidia occurred through a pathway sensitive to galactomannan and that of one single cell through a pathway sensitive to β-glucan [18]. Therefore, fungal surface polysaccharides have a key role in the DC/fungus interaction.

Transmission electronic microscopy indicated that internalization of conidia occurred predominantly by coiling phagocytosis, characterized by the presence of overlapping bilateral pseudopods that led to a pseudopodal stack before transforming into a phagosome wall. In contrast, entry of hyphae occurred by a more conventional zipper-type phagocytosis, characterized by the presence of symmetrical pseudopods which strictly followed the contour of the hyphae before fusion. However, the fate of the different forms of the fungus inside cells appeared to be quite different. Two hours after the exposure, numerous conidia were found inside DC with no evidence of conidia destruction, as opposed to hyphae that were rapidly degraded once inside cells. As killing of conidia would seem to be a necessary prerequisite to obtain efficient antigen presentation, it can be postulated that either a small number of conidia are actually degraded by mature DC thus allowing their antigen processing and presentation or, alternatively, antigens could be processed and regurgitated by other infected phagocytes and then transferred to DC for presentation. Entry of *Aspergillus* conidia through MRs resulted in the production of proinflammatory cytokines, including IL-12, up-regulation of costimulatory molecules and histocompatibility Class II antigens. IL-12 production by DC required the MyD88 pathway with the implication of distinct TLRs (TLR4 and TLR9). In contrast, coligation of CR3 with FcγR, as in the phagocytosis of hyphae, resulted in the production of IL-4/IL-10 and upregulation of costimulatory molecules and histocompatibility Class II antigens. The production of IL-10 was largely MyD88-independent [11]. Therefore, TLR collaborate with other innate immune receptors in the activation of DC against the fungus through MyD88-dependent and -independent pathways. It is of interest that TLR gene expression on DC could be affected upon fungal exposure in a morphotype-dependent manner [20] and that the TLR9 agonist CpG-ODN could convert an *Aspergillus* allergen to a potential protective antigen [23] suggesting the potential for TLR agonists to act upon the degree of flexibility of the immune recognition pathways to antigens and allergens. These results suggest that the proper manipulation of DC functioning *in vivo* may translate into beneficial effects in infections.

Experimental evidence has suggested that vaccination against *Aspergillus* through the use of fungus-pulsed DC is a feasible option [19]. The infusion of fungus-pulsed or RNA-transfected DC induced resistance to fungal infections through the induction of Th1 cells producing IFN-γ. This translated in the activation of effector phagocytes for phagocytosis and killing of the fungus and the ensuing pathogen clearance. The effect was fungus-specific, as no cross-protection was observed upon adoptive transfer of DC pulsed with either fungal species. The infusion of fungus-pulsed or RNA-transfected DC accelerated the recovery of functional antifungal responses in mice with allogeneic hematopoietic stem cell transplantation (HSCT) and affected the outcome of the infection. As DC function is impaired during the immediate period post-transplant [24,25], DC therapeutics could ameliorate recovery of resistance to fungal infections in transplant recipients. Additional findings demonstrated that *in vivo* provision of fungus-specific T cell immunity was provided by the combined action of immunogenic and tolerogenic DC. Both cDC and pDC phagocytose fungi and undergo functional maturation in response to them. However, their activation program for cytokine production was different, being IL-12 mainly produced by cDC and IL-12, IL-10 and IFN-α mainly produced by pDC. This resulted in a distinct ability for T cell priming, being Th1, Th2 and regulatory T (Treg) cells differently activated by the different DC subsets. Upon adoptive transfer *in vivo*, it was found specialization and complementarity in priming and tolerization by the different DC subsets, with pDC fulfilling the requirement for: (i) Th1/Treg antifungal priming; (ii) tolerization toward alloantigens, and (iii) diversion from alloantigen-specific to antigen-specific T cell

responses in the presence of donor T lymphocytes [26]. Thus, the remarkable functional plasticity of DC in response to fungi can be exploited for the deliberate targeting of cells and pathways of cell-mediated immunity in response to fungal vaccines.

## T helper cells

Studies on the epidemiology of invasive aspergillosis in bone marrow transplantation recipients indicated a reduced neutropenia-related infection and an increased 'late-onset' infection, concomitant with the occurrence of graft versus-host disease [27]. These findings, together with the occurrence in non-neutropenic patients [2,28], attest to the importance of specific defects in both the innate and adaptive immune effector mechanisms in the pathogenesis of the disease [29,30]. The recent evidence that, in healthy individuals and in patients surviving infection, a significant antigen-specific proliferation of IFN-γ -producing T cells occurred [30] confirms the crucial role of Th1 reactivity in the control of infection [31,32]. Two general patterns of Th activation characterize adaptive immune responses in aspergillosis. The Th1 response is associated with increased production of inflammatory cytokines IFN-γ, IL-2 and IL-12 and stimulation of antifungal effector cells (macrophages and PMNs). Alternatively, Th2 responses are associated with suppression of antifungal effector cell activity, decreased production of IFN-γ and increased concentrations of IL-4 and IL-10 which promote humoral responses (IgE) to *Aspergillus* and allergy [22]. The importance of Th1/Th2 dysregulation in the outcome of *Aspergillus* diseases in humans was recently supported by work analyzing lymphocyte responses in patients with active infection. T cell responses to *A. fumigatus* antigens were compared in healthy patients vs. patients with hematological malignancies who were receiving treatment for probable or proven invasive disease. On exposure to *Aspergillus* antigen, lymphoproliferative responses in healthy individuals exhibited a pattern of increased IFN-γ production. Patients with clinical evidence of invasive aspergillosis who were responding to antifungal therapy similarly demonstrated strong Th1 lymphoproliferative responses, with IFN-γ/lL-10 ratios greater than 1.0. Patients with stable or progressive infection on antifungal therapy, however, exhibited poor lymphocyte stimulation indexes and low IFN-γ/IL-10 ratios [30]. Together, clinical and experimental observations suggest that suppression of host Th1 $CD4^+$ lymphocyte response may contribute to the development of an unfavourable outcome of aspergillosis.

## Regulatory T cells

The inherent resistance to diseases caused by *Aspergillus fumigatus* suggests the occurrence of regulatory mechanisms that provide the host with adequate defence without necessarily eliminating the fungus or causing unacceptable levels of host damage. Moreover, Th2 cell sensitization to fungal allergens is common in atopic subjects [33], yet respiratory exposure to inhaled conidia is a tolerogenic event in most individuals. It is known that respiratory tolerance is mediated by lung DC producing IL-10 [34], which induces the development of Treg in a costimulation- and TLR-dependent fashion [35–37]. Tolerant T cells express membrane-bound transforming growth factor (TGF)-β and FoxP3 [38], the 'master control gene' for the development and function of natural $CD4^+CD25^+$ Treg cells [39].

Different types of Treg cells have been defined. Naturally occurring Treg cells originate in the thymus during the normal process of maturation and survive in the periphery as natural regulators whereas inducible or adaptive Treg cells develop from conventional $CD4^+$ T cells that are activated in conditions of blockade of costimulatory signals, presence of deactivating cytokines or drugs. $CD4^+CD25^+$ Treg cells have several modes of suppressive action at their disposal ranging from the inhibitory IL-10 and TGF-β to cell-cell contact via the inhibitory cytotoxic T lymphocyte antigen (CTLA)-4 [40]. For the naturally occurring $CD4^+CD25^+$ cells, cell-to-cell contact-dependent mechanisms have been proposed, while for the adaptive Treg cell cytokine-dependent mechanisms involving cell-surface TGF-β expression, as well as cell contact-independent mechanisms through soluble IL-10 and TGF-β have been proposed [40]. Both natural and inducible Treg cells have been described in infection, their activation occurring through both antigen-specific and non-specific mechanisms [35,37].

Distinct Treg cell populations capable of mediating anti-inflammatory or tolerogenic effects were sequentially induced by the exposure of mice to *Aspergillus* conidia. Early in infection, a population of Treg cells, expressing the same phenotype as those that control intestinal inflammation and autoimmunity [41] suppressed lung inflammatory responses to the fungus. Late in infection and, similarly in allergy, a population of Treg cells of the same phenotype as those controlling graft versus host disease [42] or diabetes [41] developed with the ability to control allergic inflammatory response to the fungus. Early in infection, $CD4^+CD25^+$ T cells were particularly increased in the lung of $B7.1^{-/-}$ mice, late in infection in the thoracic lymph nodes of $B7.2^{-/-}$ mice and were

neither detected nor increased after infection in $CD28^{-/-}$ or double-deficient $B7.1^{-/-}/B7.2^{-/-}$ mice, a finding pointing to dependency on costimulatory molecule expression. Production of IL-10 and TGF-β increased in either type of cells after infection, early $CD25^+$ cells being high producers of IL-10 and late $CD25^+$ cells of TGF-β. Therefore, phenotypically distinct $CD4^+CD25^+$ T cell populations are activated in mice exposed to *A. fumigatus*, each population being distinct from Th1 and Th2 effectors and producing IL-10 and TGF-β. Consistent with the notion that Treg cells are capable to directly affect effector Th cells and to inhibit innate immune cells through inhibitory cytokines [43] and the enzyme indoleamine 2, 3-dioxygenase (IDO) [44], late more than early $CD25^+$ Treg cells inhibited the proliferation of and IL-4 production by the corresponding $CD25^-$ population, thus suggesting that Th2 cells were inhibited. In contrast, early, more than late, Treg cells suppressed the innate effector functions of PMNs and DC, known to have a central role in the inductive and effector pathways of antifungal immunity [22]. Early Treg cells inhibited the antifungal effector and pro-inflammatory activities of PMNs, an activity occurring through both contact-dependent (CTLA-4/IDO) and -independent (IL-10) mechanisms. These results confirm previous evidence on the occurrence of PMNs capable of exhibiting suppressive antifungal effector activity through the CTLA-4/IDO-dependent mechanism [45] as well as the suppressive activity of IL-10 against the fungus [46]. With respect to DC, both early and late $CD25^+$ cells inhibited IL-12 production of lung DC in response to conidia but, upon co-culturing fungus-pulsed DC with late $CD25^+$ cells, high levels of IL-10 were also produced, a finding suggesting that, irrespective of the relative contribution of each type of cells to cytokine production, a bidirectional influence may occur between DC and Treg cells. Therefore, Th1 cell reactivity was concomitantly down-regulated in the presence of early $CD25^+$ T cells and promoted in the presence of late $CD25^+$ T cells, a finding suggesting that the capacity of early $CD25^+$ T cells (early Treg cells) to produce anti-inflammatory IL-10 sufficed to dampen the inflammatory response to the fungus while the capacity of late $CD25^+$ T cells (late Treg cells) to produce TGF-β, promoted tolerance to fungal allergy. Selective depletion of early or late Treg cells exacerbated inflammation and allergy to the fungus, whereas adoptive transfer of early or late Treg cells restored resistance to both infection and allergy, both findings indicating a causal link between resistance/susceptibility to infection and allergy and the activity of the distinct Treg cell subsets [47].

Treg cell induction and function were strictly dependent on the IFN-γ/IDO-dependent axis acting on both PMN and DC. Not only was IDO functional activity positively correlated with the suppressive activity of early Treg cells on PMNs, but IDO blockade exacerbated allergy, a finding that suggests loss of tolerogenic Treg cells. Therefore, IDO serves a crucial role in *Aspergillus* infection and allergy and is involved in both Treg cell functioning and induction. Consistent with the finding that IFN-γ is one major activating signal for IDO [44], the impaired IDO expression and functional activity observed in conditions of IFN-γ deficiency was concomitant with defective functioning of early Treg cells and defective occurrence of late Treg cells. Therefore, low levels of IFN-γ production early in infection are associated with defective activation of tolerogenic Treg cells, which links inflammatory events occurring at the early stages of the infection to subsequent allergic responses to the fungus through IFN-γ/IDO (Fig. 2).

In conclusion, regulatory mechanisms operating in the control of inflammation and allergy to the fungus are different but interdependent as the level of the inflammatory response early in infection may impact on susceptibility to allergy, in conditions of continuous exposure to the fungus. IDO has a unique and central role in this process as it participates in the effector and inductive phases of early and tolerogenic Treg cells. This may explain the beneficial effect on fungal allergy of CpG oligodeoxynucleotides [48] known to induce IDO that is found to inhibit experimental asthma [49]

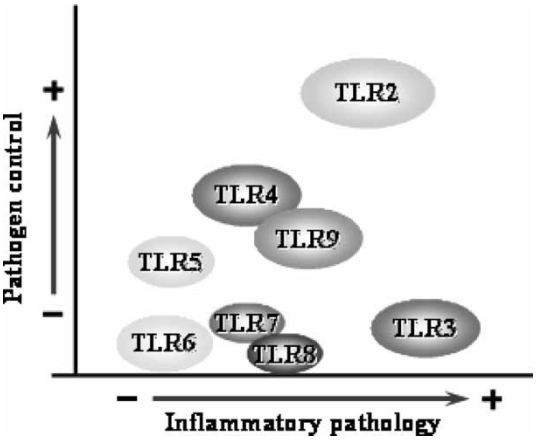


**Fig. 2** The coordinate activation of Treg cells in aspergillosis. Early in infection, anti-inflammatory Treg cells suppress antifungal inflammatory responses through an action on PMN and DC. The levels of IFN-γ produced in this early phase set the subsequent adaptive stage by conditioning the tolerogenic program of dendritic cells. This results in the occurrence, late in infection, of tolerogenic Treg cells that produce IL-10 and TGF-β and inhibit allergic Th2 cells. The IFN-γ/IDO axis has a central role in this process. See text for details.

and to have increased activity in asymptomatic atopy [50]. As an association has been reported between serum IL-10 levels and the progression of invasive aspergillosis in non-neutropenic patients [51], it is tempting to speculate that, irrespective of the underlying immunosuppressive disease state, a dysregulated Treg cell functioning may both predispose and be a surrogate marker for identifying patients at risk for *Aspergillus* infection.

## Conclusions

Immunotherapy needs to be tailored to specific immunocompromised states that predispose to opportunistic fungal infections. This demands a better understanding of the immunopathogenesis of infections. Currently available immunotherapeutic strategies for severe aspergillosis as well as other opportunistic fungal infections include the increase of phagocyte number by combination therapy with colony-stimulating factors [52]. It is conceivable that manoeuvres aimed at activating selected innate host defence pathways in phagocytes and DC and/or stimulating antigen-specific immunity (e.g., vaccines) represent future priorities for immunotherapeutics development against fungal infections. Despite some important advances in recent years, important translational issues must be resolved before a patient-tailored immunotherapeutic approach will become a reality.

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