

REVIEW

## Aspergillosis and stem cell transplantation: An overview of experimental pathogenesis studies

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### ABSTRACT

Invasive aspergillosis is a life-threatening infection caused by the opportunistic filamentous fungus *Aspergillus fumigatus*. Patients undergoing haematopoietic stem cell transplant (HSCT) for the treatment of hematological malignancy are at particularly high risk of developing this fatal infection. The susceptibility of HSCT patients to infection with *A. fumigatus* is a consequence of a complex interplay of both fungal and host factors. Here we review our understanding of the host-pathogen interactions underlying the susceptibility of the immunocompromised host to infection with *A. fumigatus* with a focus on the experimental validation of fungal and host factors relevant to HSCT patients. These include fungal factors such as secondary metabolites, cell wall constituents, and metabolic adaptations that facilitate immune evasion and survival within the host microenvironment, as well as the innate and adaptive immune responses involved in host defense against *A. fumigatus*.

### ARTICLE HISTORY

Received 19 May 2016  
Revised 24 August 2016  
Accepted 26 August 2016

### KEYWORDS



aspergillosis; aspergillus;  
immunity; pathogenesis;  
stem cell transplant;  
virulence

### Introduction

*Aspergillus fumigatus* is ubiquitous within the environment and produces abundant spores (conidia) that are disseminated by air currents. Healthy humans inhale hundreds of conidia daily without developing disease. There are multiple levels of host defense that mediate protection against this constant fungal exposure. The majority of inhaled conidia undergo mechanical elimination by mucociliary action within the airways, or are rapidly phagocytosed and killed by innate immune cells including epithelial cells and alveolar macrophages.<sup>1</sup> Conidia that escape these first lines of defense, and which undergo germination, induce epithelial cells, alveolar macrophages and dendritic cells to initiate a pro-inflammatory response through the release of cytokines and chemokines. This response leads to the recruitment of neutrophils, which exhibit potent activity against hyphae. In addition to these innate immune responses, adaptive Th1 and Th17 type responses can also mediate protection against infection.<sup>2,3</sup> Impairment in these innate or adaptive immune responses to *A. fumigatus* permits the growth of hyphae, which rapidly invade pulmonary tissues, and if unchecked, can disseminate to other deep organs. Although *A. fumigatus* conidia comprise only a

small fraction of the spores which humans inhale daily, this species accounts for the majority of invasive mold infections in immunocompromised patients.<sup>1</sup> The ability of *A. fumigatus* to grow at 37°C and to produce conidia that are small enough to penetrate deep within the airways undoubtedly contributes to the success of this pathogen,<sup>4</sup> however these properties are not unique to *A. fumigatus* and are shared by many other *Aspergillus* species and non-*Aspergillus* fungi. These observations suggest that *A. fumigatus* has developed unique virulence traits that contribute to its success as a human pathogen.

Given the array of human host defenses, invasive *Aspergillosis* (IA) is largely a disease of severely immunocompromised patients including those with primary immunodeficiencies such as chronic granulomatous disease; patients undergoing haematopoietic stem cell and solid organ transplantation; patients with hematological malignancies and prolonged neutropenia; and those receiving corticosteroid or other immunosuppressive therapies.<sup>5</sup> The burden of disease due to IA has increased significantly in the last 30 y in large part due to the increased number of patients undergoing immunosuppressive treatments for hematological malignancies and haematopoietic stem cell transplants (HSCT).<sup>6</sup>

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IA has become a leading cause of death in HSCT patients, accounting for 10% of all deaths in this population.<sup>6</sup>

In the allogeneic HSCT population, IA can occur early, during the neutropenic pre-engraftment phase, and late, during the post-engraftment period.<sup>7,8</sup> During the post-engraftment period patients are usually non-neutropenic but are immunosuppressed largely as a consequence of graft-versus-host-disease (GVHD) and the therapies given for this condition. Early IA is more common in patients undergoing myeloablative transplants due to the prolonged neutropenia resulting from the extensive chemotherapy and radiation used to destroy the native bone marrow. The rise of non-myeloablative transplantation, with a shorter neutropenic period and higher risk of GVHD, has led to a decrease in the incidence of early IA with a shift to increasing rates of late IA.<sup>9,10</sup> The late risk period associated with GVHD can last for months to years, making prophylactic and monitoring strategies challenging to implement.<sup>11</sup> Further, the sensitivity of galactomannan screening, which is commonly used for the detection of IA in neutropenic patients, is low in non-neutropenic hosts.<sup>12</sup> Thus, IA is often diagnosed at a relatively advanced stage of infection in this population, compromising the efficacy of antifungal therapy.

The pathology and immunobiology of IA is different in pre-engraftment neutropenic patients and post-engraftment non-neutropenic patients. In neutropenic patients, *A. fumigatus* infection likely begins as a bronchopneumonia, but progresses rapidly, with abundant fungal growth, early angioinvasion, thrombosis and hemorrhage often leading to fungal dissemination and eventually death.<sup>1,13-15</sup> The natural history of IA during neutropenia is therefore characterized by rapid fungal growth with low levels of inflammation. Pulmonary computed tomography (CT) imaging early in infection may reveal a bronchopneumonia, however nodular infiltrates, often surrounded by a ring of ground glass appearance (the halo sign) are more commonly detected.<sup>5,16</sup> In contrast, non-neutropenic patients receiving corticosteroids for prophylaxis or treatment of GVHD have an overabundant inflammatory response to *A. fumigatus* characterized by pyogranulomatous infiltrates and tissue necrosis, with more limited fungal growth and angioinvasion.<sup>1,14</sup> Exaggerated and uncontrolled inflammation is thought to contribute to death in this population. CT findings also include nodules but may also extend to other airway invasive type of presentations including tree-in-bud lesions and non-specific signs of bronchopneumonia.<sup>17</sup>

Given these important clinical, pathologic and radiologic differences between early and late IA, understanding the pathogenesis of aspergillosis in HSCT patients requires studies in both neutropenic and non-neutropenic experimental models. Multiple neutropenic models of IA have been

reported, including treatment of mice with chemotherapeutic agents such as cyclophosphamide or using antibody depletion of neutrophils.<sup>18,19</sup> Non-neutropenic models of IA commonly rely on the treatment of mice with corticosteroids such as cortisone acetate to induce susceptibility to infection.<sup>1</sup> These models result in experimental IA that mirrors the differences seen in human pathology between neutropenic and non-neutropenic patients.<sup>20</sup> Both of these models frequently use intranasal and intratracheal administration of high doses of conidia, however inhalational chamber methods have been described which induce reproducible infection in immunocompromised mice with an inoculum of  $10^3$  to  $10^4$  conidia per animal.<sup>19,21</sup> Although these studies have largely relied on the use of BALB/C or C57BL6 mice, a comparison of 10 inbred mouse strains using the neutropenic model of IA revealed marked differences in their intrinsic susceptibility to *A. fumigatus*.<sup>22</sup> A haplotype-based computational genetic analysis wide-association study identified a correlation between survival of these mouse strains and polymorphisms in several genes including the gene encoding for plasminogen.<sup>22</sup> Further studies testing the role of these polymorphisms in the pathogenesis of IA have not yet been reported. The use of transgenic mice in models of IA has also provided a powerful tool to study the role of host immune factors in governing defense against *A. fumigatus*, although many of these studies have been performed in immunocompetent mice infected with a very high inoculum (up to  $10^7$  conidia per animal)<sup>23</sup> and thus extrapolation to IA in HSCT requires caution. Relatively few studies have used a murine model of myeloablative HSCT-related aspergillosis in which mice are transplanted with purified haematopoietic stem cells following myeloablative irradiation before *A. fumigatus* infection.<sup>24,25</sup> Mouse models of acute leukemia and GVHD have been developed but have not been used for the study of IA.<sup>26-28</sup> Despite these limitations, studies with these experimental models have begun to shed light on the pathogenesis of IA in HSCT patients. In this review, we will examine a selection of experimental studies that highlight key factors governing the host-pathogen interaction in IA, with a focus on fungal factors that play a distinct role in neutropenic vs. non-neutropenic hosts and host factors that have been directly linked to IA susceptibility in patients undergoing HSCT.

## Fungal virulence factors

### Secondary metabolites as virulence factors and implications for HSCT

Like many molds, *A. fumigatus* can produce a variety of secreted bioactive secondary metabolites. A list of characterized *A. fumigatus* virulence factors is shown in Table 1. The epipolythiodioxopiperazine gliotoxin has

been the best studied of these mycotoxins. Gliotoxin is produced by mature hyphae of *A. fumigatus* both *in vitro*, and during human infection.<sup>29</sup> Studies *in vitro* have found that gliotoxin is a potent immunosuppressive molecule that mediates a variety of functions including inhibition of NF- $\kappa$ B activation and NADPH oxidase function, thereby preventing macrophage and neutrophil phagocytosis and oxidative killing<sup>29-32</sup>; alteration in neutrophil and macrophage cytoskeletal structure to facilitate immune evasion<sup>33</sup>; inhibition of macrophage phosphoinositide signaling protein phosphatidylinositol-3,4,5-trisphosphate, impairing phagosome formation<sup>34</sup>; and induction of leukocyte apoptosis.<sup>35,36</sup> Studies in mouse models have been helpful in understanding the relative importance of these various functions in the pathogenesis of IA. Initial studies in neutropenic models of IA found that *A. fumigatus* gliotoxin-deficient mutants are fully virulent.<sup>37-39</sup> However, in a non-neutropenic mouse model, gliotoxin-deficient mutants display attenuated virulence and induce lower levels of apoptosis in neutrophils surrounding fungal lesions.<sup>40,41</sup> Collectively, these studies suggest that gliotoxin contributes to virulence largely through the induction of neutrophil apoptosis and thus this toxin plays little role in the pathogenesis of IA in neutropenic hosts such as HSCT patients pre-engraftment.

It is likely that secondary metabolites other than gliotoxin also contribute to virulence. Support for this hypothesis comes from studies of the global regulator of secondary metabolite production LaeA. Deletion of *laeA* leads to a near complete repression of all secondary metabolite biosynthetic gene clusters in *A. fumigatus*, including the genes required for the production of

gliotoxin.<sup>42</sup> In contrast to gliotoxin-deficient mutants, the  $\Delta$ *laeA* mutant is attenuated in virulence in both neutropenic and non-neutropenic murine models of IA.<sup>42-44</sup> While the attenuated virulence of the LaeA-deficient mutant in non-neutropenic mice may be due in part to decreased gliotoxin expression, this mutant is much more attenuated in virulence than gliotoxin-deficient strains, suggesting that LaeA controls other secondary metabolites important for virulence. This hypothesis is supported by the observation that the LaeA-deficient mutant is also virulent in neutropenic mice in which gliotoxin is dispensable for virulence.<sup>42</sup> LaeA has been observed to mediate *A. fumigatus*-dependent inhibition of angiogenesis in a neutropenic mouse model by both gliotoxin-dependent and -independent factors.<sup>43</sup> It has been suggested that inhibiting angiogenesis may help sequester the fungus from host defenses and antifungals to promote infection. Importantly however, LaeA also governs other fungal processes in addition to the regulation of secondary metabolite production, including the expression of hydrophobins and of *alb1*, a gene required for synthesis of conidial pigment.<sup>44</sup> As detailed below, both of these factors have been reported to play a role in the pathogenesis of IA in experimental mouse models. Studies of individual secondary metabolites are likely required to elucidate their role in the pathogenesis of IA and to determine their potential as therapeutic targets in the context of HSCT-associated IA.

### Cell wall components that play an important role in the pathogenesis of IA

As the site of first contact between the host and fungus during infection, the fungal cell wall plays a key role in the pathogenesis of IA. Although a comprehensive discussion of the role of the fungal cell wall components in virulence is beyond the scope of this review, a number of more recent experimental findings will be highlighted.

#### Components of the cell wall of conidia

Resting conidia of *A. fumigatus* are extremely hydrophobic due to the presence of an organized layer of immunologically inert rodlet proteins (RodA) that cover and conceal the underlying cell wall polysaccharides.<sup>45</sup> Studies *in vitro* have demonstrated that this rodlet layer confers a variety of phenotypes on resting conidia including rendering them adhesive to host macromolecules and masking  $\beta$ -glucans and other polysaccharides from recognition by host macrophages and dendritic cells.<sup>45-47</sup> Studies in mouse models, however, have yielded conflicting results. In immunocompetent mice, loss of RodA results in a dramatic increase in pulmonary inflammatory responses, and impaired virulence in a corneal

**Table 1.** Fungal virulence factors reviewed in this paper and their role in virulence in immunocompetent, neutropenic and non-neutropenic mouse models. – indicates that the contribution of the indicated factor to virulence in this experimental model is not known.

Virulence Factor	Mouse Model and Contribution to Virulence		
	Immunocompetent	Neutropenic	Non-neutropenic
Gliotoxin	—	NO	YES
LaeA	—	YES	YES
RodA	YES	—	—
Melanin/Alb1	YES	—	—
Ags1, 2, 3	YES	YES	—
GAG/Uge3	—	YES	YES
RbhA	—	YES	YES
SidA, C, D, F, G	—	YES	YES
HapX	—	YES	YES
AcuM	—	—	YES
AcuK	—	—	YES
SrbA	—	YES	YES
SrbB	—	—	YES
RbdA	YES	—	YES
HorA	—	YES	YES

model of *A. fumigatus* infection.<sup>46</sup> However, conidia deficient in RodA are fully virulent in a corticosteroid treated, non-neutropenic mouse model of pulmonary IA.<sup>45</sup> Studies in neutropenic mice have not yet been reported. Thus, although RodA clearly plays a role in preventing potentially deleterious pulmonary inflammatory responses to daily conidial exposure in healthy individuals, its role in the pathogenesis of IA in HSCT patients remains unresolved.

*A. fumigatus* conidia are heavily pigmented due to the presence of cell wall melanin. Studies *in vitro* have identified a number of roles for melanin in the host-pathogen interaction including contributing to cell wall integrity, modulating macrophage cytokine responses, and inhibition of phagolysosome acidification of alveolar macrophages, monocytes and neutrophils.<sup>48-52</sup> The mechanisms by which melanin interacts with phagolysosomes to contribute to disease are beginning to be elucidated. It has been suggested that melanin interferes with LC3-associated phagocytosis (LAP), an Atg5-dependent autophagy pathway that promotes fungal killing.<sup>53,54</sup> When Atg5 was inactivated in macrophages and murine haematopoietic cells, the virulence of a melanin-deficient *A. fumigatus* strain was restored. Consistent with these *in vitro* observations, conidia melanin production is essential for normal virulence.<sup>55,56</sup> Mutant *A. fumigatus* strains lacking the conidial pigmentation gene *alb1* (also known as *pksP*) are significantly attenuated in virulence in immunocompetent mice.<sup>57</sup> It is therefore likely that melanin contributes to the establishment of both early and late IA in HSCT patients.

Although  $\alpha$ -1,3-glucans are an important component of both conidia and hyphae, recent studies have highlighted a role for these glycans in the cell wall of conidia during experimental IA. Synthesis of  $\alpha$ -1,3-glucans is mediated by one of 3 glucan synthases: *ags1*, *ags2* and *ags3*.<sup>58,59</sup> Single deletions of each of these genes is associated with minimal changes in cell wall composition, however loss of all 3 genes results in a complete absence of  $\alpha$ -1,3-glucan synthesis, and extensive structural modification of the cell wall.<sup>60</sup> Absence of  $\alpha$ -1,3-glucans is associated with the production of an amorphous layer of glycoproteins that covers the normal rodlet layer, leading to enhanced recognition of conidia by macrophages.<sup>61</sup> The  $\alpha$ -1,3-glucan deficient mutant is markedly impaired in virulence in both immunocompetent and neutropenic models of aspergillosis, possibly through enhanced elimination of conidia by pulmonary macrophages.<sup>61</sup> Studies in corticosteroid-treated mice have not been reported. The dramatic effect on virulence in a neutropenic host suggests the possibility that inhibition of  $\alpha$ -1,3-glucan synthesis could be an effective antifungal strategy in the future for the treatment of early IA in the HSCT population.

### Components of the hyphal cell wall that play a role in virulence

Hyphae of *A. fumigatus* are covered in a layer of galactosaminogalactan (GAG), a partially deacetylated heteropolymer of  $\alpha$ -1,4 linked galactose and N-acetyl galactosamine.<sup>61-63</sup> GAG has been described to mediate a number of virulence-associated functions *in vitro* including mediating adhesion and biofilm formation<sup>64</sup>; masking  $\beta$ -1,3-glucan from host recognition by dectin-1 on dendritic cells; mediating resistance to neutrophil extracellular traps<sup>65</sup>; inducing natural killer cell-mediated neutrophil apoptosis<sup>66</sup>; and the induction of expression of the immunosuppressive cytokine IL-1RA.<sup>67</sup> In contrast to gliotoxin, *in vivo* studies using GAG-deficient mutants found that GAG effects on virulence are more marked in neutropenic mice,<sup>64</sup> suggesting that neutrophil-independent functions of GAG are more important in mediating virulence. Conversely, it was found that increasing GAG expression in the naturally GAG-deficient species *Aspergillus nidulans* enhances virulence in non-neutropenic but not neutropenic mice.<sup>65</sup> In this system, the predominant role of GAG is to mediate resistance to killing by neutrophil extracellular traps. Further studies are required to reconcile these findings and better understand the role of GAG in virulence in neutropenic and non-neutropenic hosts.

### Other fungal factors involved in the pathogenesis of IA

Adaptation to the host environment is a critical factor for the survival of *A. fumigatus* during infection. For example, *A. fumigatus* mutants defective in nitrogen metabolism, such as the  $\Delta$ *rbhA* mutant, exhibit significantly reduced growth and virulence in both neutropenic and non-neutropenic mouse models of infection.<sup>68,69</sup> The ability to acquire micronutrients such as iron is similarly critical for fungal growth *in vivo*. Not surprisingly, siderophore biosynthesis has also been implicated in virulence in both neutropenic and non-neutropenic mouse models.<sup>70-73</sup> The *A. fumigatus*  $\Delta$ *sidA* mutant, which is completely deficient in iron siderophore synthesis, grows poorly in low iron conditions and within alveolar macrophages and is completely avirulent.<sup>73</sup> Attenuated virulence has also been reported in other strains of *A. fumigatus* with mutations in the siderophore synthesis pathway and with other mutations affecting iron metabolism, including HapX, AcuM, and AcuK.<sup>72,74-76</sup> Taken together, these studies strongly suggest that the ability of *A. fumigatus* to acquire and utilize iron from the host is essential for pathogenicity. This hypothesis is supported by the observation that iron overload within HSCT patients is a risk factor for the development of IA.<sup>77,78</sup>



Recent studies have also implicated the ability of *A. fumigatus* to adapt to hypoxic conditions as a critical factor in the development of IA. During *A. fumigatus* infection, the organism grows within a hypoxic microenvironment resulting from a number of factors including impaired blood supply from thrombosis and tissue necrosis, inhibition of angiogenesis, and increased oxygen consumption by host inflammatory cells.<sup>79</sup> Fungal adaptation to hypoxia therefore is critical for pathogenicity.<sup>80</sup> *A. fumigatus* SrbA and SrbB, members of the highly conserved sterol regulatory element binding protein (SREBP) family of transcription factors, have been reported to play critical roles in fungal adaptation to hypoxia.<sup>81,82</sup> *In vitro*, SrbA is required for normal hyphal growth and cell wall morphology under hypoxic conditions, and increases ergosterol biosynthesis, thereby contributing to azole antifungal resistance.<sup>81</sup> Interestingly, SrbA also regulates iron acquisition in response to hypoxia and low iron conditions by increasing siderophore biosynthesis.<sup>83</sup> SrbB mediates similar functions to SrbA but also regulates heme biosynthesis, as well as carbohydrate and sterol metabolism under hypoxic conditions.<sup>82</sup> Mutants deficient in SrbA display significantly reduced growth and are avirulent in both neutropenic and non-neutropenic mouse models, while SrbB-deficient mutants are markedly reduced in virulence in a non-neutropenic mouse model.<sup>81-83</sup> The virulence of SrbB-deficient mutants in neutropenic mouse models has not been reported. Notably, mutants deficient in both SrbA and SrbB are more impaired in growth and virulence than mutants deficient in either protein.<sup>82</sup> This observation suggests that SrbA and SrbB regulate both shared and distinct genes that are crucial to fungal metabolism and virulence. Another protein in the SREBP family, SrbC, remains to be characterized. Roles for other proteins in the regulation of the hypoxic response are now emerging. The iron metabolism regulators HapX and SreA have been found to play a role in hypoxia adaptation.<sup>83,84</sup> Most recently, the signal peptide peptidase SppA, the protease RbdA, and the hypoxia-induced dehydrogenase HorA have also been linked to hypoxia adaptation and virulence, possibly through interactions with the Srb pathways.<sup>80,85,86</sup> Together these data suggest a model whereby the ability of *A. fumigatus* to thrive in hypoxic microenvironments is a critical factor in the pathogenesis of IA and suggest a potential role for hyperbaric oxygen therapy for IA in HSCT patients.

Recent advances in microfluidics have provided new tools for the *in vitro* study of the effect of micro-environmental conditions on fungal growth and secondary

metabolite production. A micrometabolomics platform has been developed which couples a microfluidic solid or liquid culture system to liquid chromatography-mass spectrometry for the profiling and discovery of secondary metabolite production in response to environmental variations.<sup>87</sup> Use of this system revealed a clear relationship between *A. fumigatus* colony diameter and the secondary metabolite profile produced by the active culture that was independent of biomass.<sup>87</sup> Similarly, marked differences were observed in the metabolite profile of *A. fumigatus* grown in blood as compared with grown in standard culture media.<sup>87</sup> A similar system has been used to study the dynamics of germination in the pathogenic yeast *Cryptococcus neoformans*.<sup>88</sup> The use of this and other similar micro-systems may provide a useful tool to improve our understanding of the importance of the microenvironment on the expression and function of virulence factors of *A. fumigatus*.

#### **Factors underlying the virulence of non-*fumigatus* *Aspergillus* species**

Although *A. fumigatus* accounts for the majority of cases of IA, other species such as *A. terreus* and *A. flavus* can also cause disease in HSCT patients. There are relatively few studies probing the host-pathogen interactions of these *Aspergillus* species during infection. The virulence of *A. flavus* has been evaluated in intravenous models of disseminated IA in both immunocompetent and neutropenic mice. In intravenous infection models, *A. flavus* was reported to exhibit increased virulence as compared with *A. fumigatus*.<sup>89</sup> However, it has been postulated that the larger spore size of this species limits its penetration of the lower airways, thus reducing the ability of this species to establish infection. The virulence and natural history of *A. terreus* infection in neutropenic and non-neutropenic mouse models have also been compared. In neutropenic mice, infection with *A. terreus* resulted in a degree of mortality that was similar to previous reports of *A. fumigatus* infection in the same model.<sup>90</sup> In contrast, *A. terreus* exhibited attenuated virulence in corticosteroid-treated mice as compared with prior reports of *A. fumigatus* in this model.<sup>90</sup> Interestingly, in both models, fatty degeneration of hepatocytes was noted, a finding not previously reported in *A. fumigatus* infection.<sup>90</sup> Confirmation of these findings awaits a direct head-to-head comparison of *A. terreus* and *A. fumigatus* infection in these models. Unlike studies of pathogenic yeast, the heterologous expression of putative *A. fumigatus* virulence factors in less virulent

*Aspergillus* species has only been reported in a single study.<sup>65</sup> In this work, detailed above, heterologous expression of an *A. fumigatus* glucose epimerase was used to enhance the production of cell wall galactosaminogalactan in *A. nidulans*, and was associated with increased virulence in neutropenic mice.<sup>65</sup> Heterologous expression of non-*fumigatus* *Aspergillus* virulence factors in *A. fumigatus* and the converse holds promise for the study of other species-specific virulence factors in mouse models.

### Host response to aspergillosis

Our understanding of the host response to *A. fumigatus* has greatly increased in recent years. As an exhaustive review of fungal immunology studies is beyond the scope of this review, we will highlight advances in our understanding of host defense against IA, with an emphasis on immune factors that have been linked to susceptibility of HSCT populations to this infection. A general schematic of host immune factors involved in defense against *A. fumigatus* is shown in [Figure 1](#).

### Innate immune defenses against invasive aspergillosis in HSCT patients

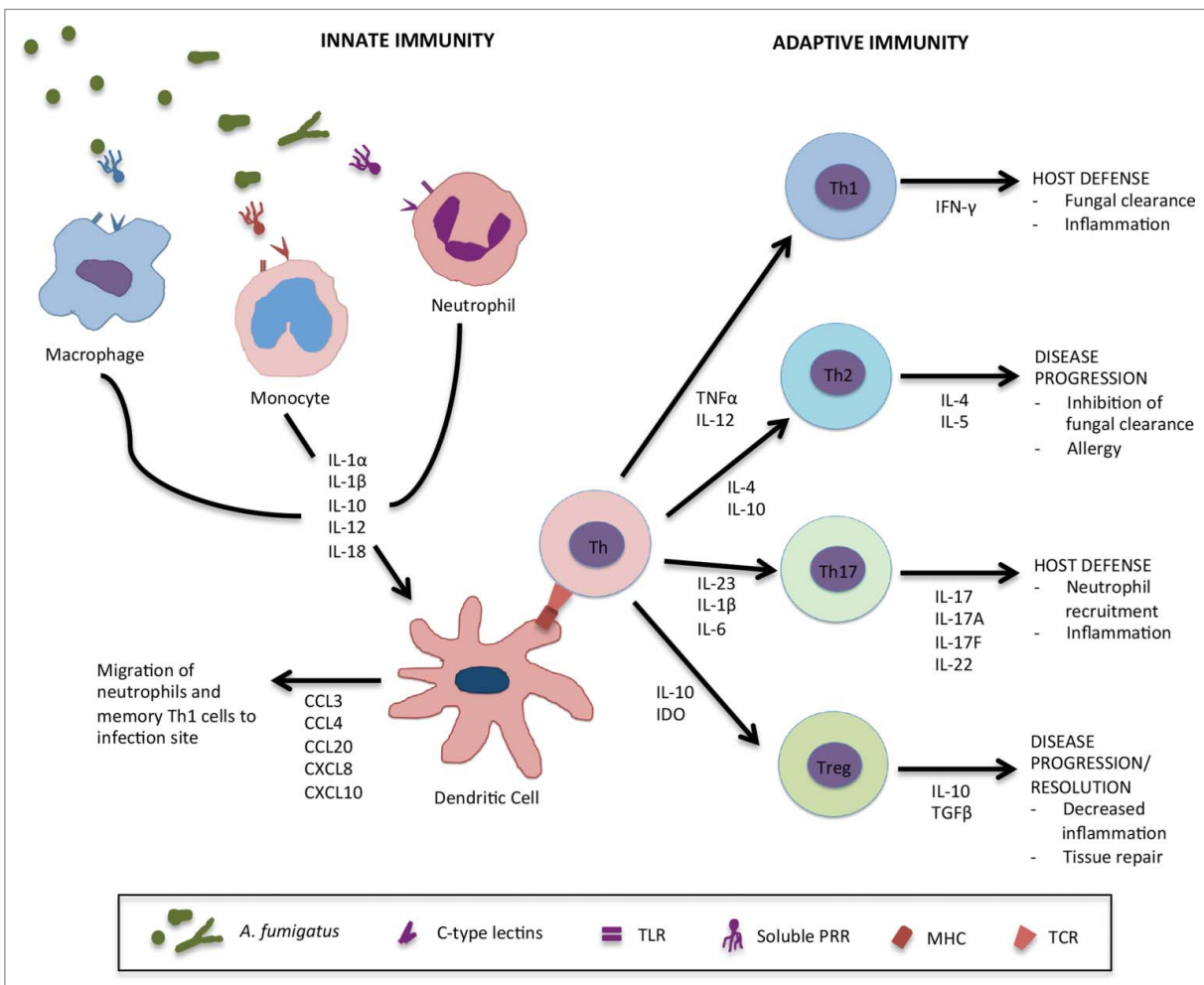
Conidia that escape elimination by pulmonary mucociliary action or by alveolar macrophages shed their hydrophobin coat and undergo germination. Recognition of these germinated conidia and hyphae by the innate immune system is critical for the control of infection. Effective innate immune response to early IA begins with the recognition of fungal elements by cell surface pattern recognition receptors (PRRs) such as the C-type lectins dectin-1 and dectin-2; members of the toll-like receptor (TLR) family; and soluble factors such as pentraxin-3, surfactant proteins A and D (SP-A, SP-D) and mannose-binding lectin (MBL) ([Figure 1](#)).<sup>11</sup> Effective fungal recognition leads to the activation of inflammatory programs including the NF $\kappa$ -B pathway and the NLRP3 inflammasome resulting in secretion of a variety of pro-inflammatory cytokines and chemokines by epithelial cells, inflammatory monocytes, dendritic cells (DCs) and alveolar macrophages ([Figure 1](#)). This cytokine environment contributes to the recruitment and activation of effector cells such as neutrophils which mediate fungal killing.<sup>11</sup> Polymorphisms in a number of key genes involved in mediating these innate responses to *A. fumigatus* have been identified in HSCT donors and recipients.<sup>91,92</sup>

### Cellular pattern recognition receptors

Several cellular PRRs have been implicated in the recognition of *A. fumigatus*, of which dectin-1 and members of the TLR family have been best studied. Dectin-1 is a C-type lectin expressed by macrophages and DCs that recognizes the *A. fumigatus* cell wall constituent  $\beta$ -1,3-glucan.<sup>93</sup> Interaction of dectin-1 with *A. fumigatus* conidia promotes their phagocytosis, enhances macrophage activation, and induces proinflammatory responses including the secretion of tumor necrosis factor (TNF).<sup>94</sup> Dectin-1 mediates recruitment of the autophagy protein LC3 II to phagosomes in monocytes, a process that is inhibited by corticosteroids.<sup>95,96</sup> Immunocompetent mice deficient in dectin-1 are more susceptible to *A. fumigatus* infection.<sup>97-99</sup> In an experimental HSCT mouse model investigators observed that dectin-1 deficient mice are more susceptible to IA than wild-type mice following transplantation with wild-type stem cells. Dectin-1 deficient mice transplanted with stem cells from donors deficient in dectin-1 exhibit the highest susceptibility to IA, while wild-type mice transplanted with dectin-1 deficient stem cells do not exhibit increased susceptibility to infection.<sup>98</sup> These findings suggest that expression of dectin-1 by non-haematopoietic cells may also play an important role in mediating protection against IA during HSCT.

Experimental studies also suggest that dectin-1 may hold promise for therapy of IA. Transient transfection experiments in neutropenic mice have demonstrated protective effects of augmenting expression of native dectin-1 or of a fusion protein consisting of the extracellular domain of dectin-1 linked to the Fc portion of murine immunoglobulin G1.<sup>100,101</sup> Human studies of dectin-1-based therapeutics have not been reported.

Cell-surface expressed TLRs also contribute to the recognition of *A. fumigatus*. TLR2 and TLR4, which signal through the adaptor protein MyD88, enhance pro-inflammatory cytokine production in response to *A. fumigatus*.<sup>49,102</sup> TLR2 forms a heterodimer with TLR1 and TLR6 in mouse cells, but only TLR1 in human cells has been hypothesized to play an accessory role to dectin-1 in the induction of TNF responses to *A. fumigatus*.<sup>102,103</sup> Two studies have examined the susceptibility of TLR2-deficient mice to infection with *A. fumigatus*. In both studies, TLR2 $^{-/-}$  mice developed higher pulmonary fungal burdens during infection; however increased mortality in neutropenic TLR2 $^{-/-}$  mice was only observed in one of these 2 studies.<sup>104,105</sup> In this neutropenic mouse model, TLR4 $^{-/-}$  mice were more susceptible to *A. fumigatus* infection than TLR2 $^{-/-}$  mice.<sup>105</sup> The role of these TLRs in the pathogenesis of non-neutropenic IA has not yet been studied.



**Figure 1.** Host immune response against *A. fumigatus*. Conidia which enter the lungs and escape airway epithelial cells are recognized by innate immune effector cells via cell surface pattern recognition receptors (PRRs) such as dectin-1 or toll-like receptors (TLRs) and soluble factors such as pentraxin-3 (PTX-3), surfactant proteins A and D (SP-A,D) or mannose-binding lectin (MBL). Alveolar macrophages recognize and clear conidia leading to the activation of inflammatory pathways and the secretion of various proinflammatory cytokines by epithelial cells, other macrophages and dendritic cells. The cytokine environment contributes to the recruitment of other innate immune effectors such as inflammatory monocytes and neutrophils which mediate fungal killing of conidia that have escaped initial recognition and germinated into hyphae and further stimulate proinflammatory responses. Dendritic cells (DCs) are the link between the innate and adaptive immune responses to *A. fumigatus*. During infection and after interaction with other innate effectors, they secrete various chemokines such as CCL3, CCL4, CCL20, CXCL8 and CXCL10 to allow migration of more inflammatory cells to the site of infection. They also interact with immature helper T cells (Th) through the major histocompatibility complex (MHC) and T cell receptor (TCR), resulting in the secretion various cytokines driving protective Th1 and Th17 responses against *A. fumigatus*. They can also inhibit Th2 responses which favor disease progression and allergy. Regulatory T cells are producers of IL-10 which is linked to disease progression in IA.

The intracellular PRR TLR9, which recognizes unmethylated CpG DNA, traffics to phagosomes containing *A. fumigatus*.<sup>106,107</sup> It has been suggested that TLR9 recruitment to phagolysosomes acts to inhibit inflammatory responses and prevent over-exuberant inflammation. In support of this hypothesis, neutrophil-depleted TLR9<sup>-/-</sup> mice displayed reduced pulmonary fungal growth when infected with resting conidia, and prolonged survival when infected with swollen conidia. A more recent study suggests that in non-neutropenic hosts TLR9 may play a different role and induce TNF

production and neutrophil recruitment via calcineurin-NFAT mediated signaling.<sup>108</sup> This observation raises the possibility that loss of TLR9 signaling may play an important role in the susceptibility of non-neutropenic HSCT patients to IA in the post-engraftment phase.

Studies in human patients have confirmed the importance of many of these PRRs in mediating immunity to *A. fumigatus*. HSCT patients who received allogeneic stem cells with TLR1, TLR4, TLR6 and dectin-1 polymorphisms have significantly increased susceptibility to IA.<sup>98,109-111</sup> One of the best studied of these

mutations is the dectin-1 Y238X polymorphism, which results in the production of a truncated dectin-1 protein that is unable to localize to the cell membrane and mediate fungal recognition.<sup>98</sup> The presence of this mutation in HSCT donors or recipients is associated with a significantly increased susceptibility to IA.<sup>98</sup> Interestingly, normal patients who received donor stem cells with the Y238X polymorphism had an increased risk of IA in both the early (0–30 days) and late (30–120 days) stages after transplantation while recipients carrying the polymorphism receiving normal donor cells largely developed IA very late (after 120 days) following transplantation. As was reported in mouse models, the worst prognosis was observed in cases in which both donor and recipient carried this dectin-1 polymorphism.<sup>98</sup> Furthermore, significantly lower levels of proinflammatory cytokines IFN $\gamma$ , IL-10, IL-1 $\beta$ , IL-6 and IL-17A were detected in the serum of patients with the Y238X polymorphism during *Aspergillus* infection, consistent with a role for dectin-1 in mediating innate host defense against *A. fumigatus*.<sup>98</sup> These observations suggest the possibility that mapping of these polymorphisms may allow identification of subpopulations of patients at higher risk for IA that may benefit from more aggressive screening or prophylactic strategies.

#### **Soluble factors that are critical for defense against invasive aspergillosis**

Experimental studies in transgenic mice have identified a role for the soluble PRR pentraxin-3 in host defense against *A. fumigatus*. Pentraxin-3 binds to fungal galactomannan and enhances the uptake and killing of conidia by macrophages as well as activation of DCs.<sup>97,112,113</sup> Immunocompetent mice deficient in pentraxin-3 were more susceptible to *A. fumigatus* infection and failed to mount a protective Th1 adaptive immune response to this organism.<sup>113</sup> Administration of recombinant pentraxin-3 protects against *A. fumigatus* infection in a murine model of allogeneic transplantation in which mice were challenged with *A. fumigatus*.<sup>114</sup> In this model, treatment with pentraxin-3 results in accelerated recovery of pulmonary phagocytic cell populations and Th1 lymphocytes and was synergistic with amphotericin B therapy.<sup>115,116</sup> Consistent with these experimental studies, it has been reported that HSCT recipients transplanted with donor cells containing a polymorphism in the pentraxin-3 gene *PTX3*, displayed a higher incidence of IA.<sup>117</sup> The presence of this polymorphism was associated with reduced levels of pentraxin-3 production by neutrophils and a functional defect in neutrophil phagocytosis and killing of *A. fumigatus in vitro*.<sup>117</sup> Collectively these data suggest a possible role for screening of HSCT patients with IA in order to identify pentraxin-3 deficient

patients that may benefit from replacement therapy. Genome-wide association studies are underway to explore host susceptibility to IA based on genetic defects, which can be used as a basis for clinical trials involving medical intervention based on patient genetic screening and immune profiling.<sup>118</sup>

Two recent experimental studies have examined the role of IL-1 $\alpha$  and inflammasome-dependent IL-1 $\beta$  in the pathogenesis of IA.<sup>119,120</sup> In neutropenic mice, the processing of IL-1 $\beta$  by the NLRP3 inflammasome is critical for defense against pulmonary challenge by *A. fumigatus*.<sup>119</sup> In contrast, a more modest role for inflammasome-dependent IL-1 $\beta$  was reported in immunocompetent mice challenged with a high dose of conidia.<sup>121</sup> In this model, IL-1 $\alpha$  played a dominant role in enhancing the recruitment and activity of neutrophils.<sup>121</sup> The role of IL-1 $\beta$  and IL-1 $\alpha$  in the pathogenesis of IA in non-neutropenic immunocompromised hosts remains unstudied. Interestingly, a polymorphism in NLRP3 (rs35829419, Q705K) was found to be associated with the development of IA in HSCT patients when present in either the donor or recipient.<sup>122</sup> Surprisingly, however, this polymorphism was reported to be associated with increased activation of the inflammasome and IL-1 $\beta$  production. Further studies will be required in human populations to better understand the importance of the inflammasome in mediating antifungal defense during HSCT.

#### **Epithelial cells in the innate defense against *A. fumigatus* infection**

Successful infection of pulmonary epithelial cells by *A. fumigatus* conidia and hyphae has long been hypothesized to play an important role in the pathogenesis of invasive aspergillosis.<sup>123–126</sup> In a mouse model of chemotherapy-induced neutropenia, treatment of mice with an inhaled synergic combination of TLR2, 6 and 9 agonists induced protection against *A. fumigatus*, by enhancing mucosal and epithelial cell responses.<sup>28</sup> Similar protection was observed against other pathogens including *Pseudomonas aeruginosa*, suggesting that enhancing epithelial cell resistance to infection may be a useful strategy to reduce mortality due to opportunistic pneumonia in the HSCT population.

#### **The adaptive immune response to aspergillosis in HSCT patients**

The importance of cellular and acquired immunity in the protection against *A. fumigatus* infection is well established in mouse models of IA. Early studies in these models showed that production of Th1-type cytokines such as IFN $\gamma$ , TNF $\alpha$  and IL-12 is associated with



resistance to disease, while the production of Th2 cytokines such as IL-4 is associated with disease progression (Figure 1).<sup>127</sup> The association of a Th2 cytokine environment with IA was shown through early experiments using transgenic mice deficient in IL-4. In both immunocompetent and neutropenic mouse models, IL-4<sup>-/-</sup> mice were protected against IA.<sup>128</sup> Interventional studies with adoptive transfer of immune cells have highlighted the importance of Th1-mediated immunity in resistance to *A. fumigatus* infection. Vaccination of immunocompetent and neutropenic mice with T cells and DCs pulsed with *Aspergillus* antigens in order to induce Th1 mediated resistance was associated with enhanced survival following fungal challenge.<sup>129,130</sup> Studies of human DCs *in vitro* have found that these cells produce chemokines and other ligands CCL3, CCL4, CXCL8, CXCL10 and CCL20 that stimulate migration of polymorphonuclear cells (PMNs) and memory Th1 cells to the site of infection aiding in host resistance (Figure 1).<sup>131,132</sup> Interestingly in humans, polymorphisms in chemokine ligand CXCL10 resulting in decreased DC CXCL10 production have been associated with increased risk of IA in HSCT patients.<sup>22,133</sup> Further studies, using DC vaccines to promote Th1 immunity, have shown promise in HSCT mouse models.<sup>134,135</sup> In these studies, adoptive transfer of DCs pulsed with *A. fumigatus* conidia or transfected with *A. fumigatus* conidial RNA induced the production of IFN $\gamma$  producing Th1 cells in mice following HSCT and increased resistance to *A. fumigatus* challenge.<sup>134</sup> Interestingly, resistance to IA was not observed when DCs were pulsed with *A. fumigatus* hyphae. In other studies, HSCT mice treated with *Aspergillus* specific Th1 cells by adoptive transfer also exhibited increased resistance to *A. fumigatus* infection.<sup>134,136</sup> More recently, the adoptive transfer of transgenic T-cells engineered to express a chimeric antigen receptor which incorporated the extracellular portion of the  $\beta$ -glucan receptor, dectin-1, was reported to reduce fungal burden in a neutropenic model of IA.<sup>137</sup> Building on these experimental studies, the role of fungal specific T-cells in the treatment or prevention of IA in HSCT patients is an area of intense clinical interest, and holds great potential for the management of this infection, although significant challenges remain in translating these experimental techniques to clinical practice.

As with the Th2 cytokine IL-4, the immunosuppressive cytokine, IL-10, produced by regulatory T cells, enhances susceptibility to IA (Figure 1). In non-neutropenic mice pre-treated with corticosteroids, high levels of pulmonary IL-10 expression were associated with IA mortality.<sup>138,139</sup> In both immunocompetent and non-neutropenic mouse models, IL-10<sup>-/-</sup> mice were better able to control fungal infections and survived longer

when challenged with lethal doses of *A. fumigatus*.<sup>138,139</sup> Polymorphisms in the IL-10 promoter gene have been identified in HSCT patients and have been linked to GVHD and susceptibility to IA.<sup>140-142</sup> IL-10 promoter polymorphisms resulting in increased transcript levels are associated with increased susceptibility to both GVHD and IA,<sup>141</sup> while patients with promoter polymorphisms resulting in low to undetectable transcript levels were much more tolerant to *A. fumigatus* infection and disease.<sup>142</sup> Further study of IL-10 promoter polymorphisms in humans may be useful both to identify HSCT patients susceptible to IA and to understand the role this cytokine plays in host immunity.

The role of the IL-23 – IL-17 axis in the pathogenesis of IA is somewhat controversial. IL-23 produced by macrophages and DCs in response to *A. fumigatus* leads to Th17 polarization and IL-17 production during experimental infection.<sup>3,143</sup> An initial study reported that neutralization of IL-17 and IL-23 in immunocompetent mice improved the outcome of IA and suggested that the IL-23/IL-17 axis inhibits protective Th1 mediated responses while inducing damaging host inflammatory responses.<sup>144</sup> However, other studies using anti-IL-17 antibodies have reported that neutralization of IL-17A renders mice more susceptible to *A. fumigatus* infection.<sup>93</sup> Similarly, in an experimental model of pulmonary *A. fumigatus* colonization in immunocompetent mice, clearance of the fungus from the airways was associated with the development of Th17 responses,<sup>145</sup> and IL-17 production by neutrophils has been found to be protective in a model of *A. fumigatus* keratitis.<sup>144</sup> Similar conflicting results have been reported from human studies. *A. fumigatus* was a weak inducer of IL-17 production in human T-cells *in vitro*, and low levels of IL-17 were detected in the bronchoalveolar lavage (BAL) of patients with IA.<sup>146,147</sup> Active down regulation of IL-17 production through fungal tryptophan metabolism and kynurenine production was suggested as a mechanism whereby *A. fumigatus* evades Th17 responses.<sup>146</sup> Polymorphisms in IL-23 but not IL-17A and IL-17F genes were associated with lower rates of IA in a cohort analysis of T-cell depleted allogeneic HSCT patients.<sup>148</sup> Overall, the role of the Th17 response and IL-17 in IA remains poorly understood, and further studies are required to evaluate the utility of treatment strategies targeting this immune axis.

Finally, although *Aspergillus* antibody responses have largely been viewed as non-protective in IA, several studies using carbohydrate antigens and anti-carbohydrate monoclonal antibodies have challenged this dogma. Active vaccination of mice with  $\beta$ -glucan conjugated to diphtheria toxoid protected immunocompetent mice from intravenous challenge with *A. fumigatus*, although protection of immunosuppressed mice and efficacy in

pulmonary disease were not evaluated.<sup>149</sup> In a more recent study, administration of a monoclonal IgM antibody targeting a sialylated  $\beta$ -1,3-linked oligosaccharide was found to protect mice from intravenous challenge with *A. fumigatus*.<sup>150</sup> Excitingly, treatment with this antibody also improved the survival of neutropenic mice infected intratracheally with *A. fumigatus* though not when these mice were infected intravenously. The mechanism by which this monoclonal antibody mediates protection in neutropenic mice is unclear, but may involve complement and/or enhancing phagocytic killing by non-neutrophil leukocytes. The findings of these studies suggest the exciting possibility that passive therapy with anti-*Aspergillus* antibodies could be an effective strategy for the prevention of IA during HSCT.

## Conclusion

The pathogenesis of IA is governed by a complex interplay between environmental conditions, fungal virulence factors and the host immune response. Patients undergoing HSCT encompass a range of different physiological and immunological environments as a function of their underlying hematological disease; the chemotherapy and conditioning regimens administered pre-transplant; the engraftment status and degree of neutropenia; the presence of secondary infections that modulate host immunity such as CMV; and the degree of GVHD and the immunosuppressive therapies used for this condition. Many fungal virulence factors and host immune parameters are therefore likely to play an important role in pathogenesis in select groups of patients at specific times during their course of treatment. This level of complexity is a major challenge for the study of host-pathogen interactions in this population and will necessitate the validation of experimental results in multiple animal models as well as human populations. The development of robust and practical animal models for leukemia, HSCT and GVHD will be critical tools for the advancement of this field. Nonetheless, there is cause for optimism. Human genetic association studies have validated many of the findings in mouse studies of *Aspergillus* immunity. The identification of these key host determinants of susceptibility may lead to pre-transplant screening algorithms that can identify very high-risk patients who would be candidates for aggressive surveillance or antifungal prophylactic strategies and the development of novel immunotherapies for the prevention and treatment of IA. Similarly, the recent identification of novel fungal virulence factors may lead to the development of new antifungal strategies for this important disease. Collectively, these advances hold promise for an era of new therapies

targeting the host-fungal interphase in order to reduce mortality due to IA in this vulnerable population.

## Abbreviations

BAL	bronchoalveolar lavage
CT	computed tomography
DC	dendritic cell
GAG	galactosaminogalactan
GVHD	graft-vs-host disease
HSCT	haematopoietic stem cell transplantation
IA	invasive aspergillosis
MBL	mannose-binding lectin
PMN	polymorphonuclear cell
PRR	pattern recognition receptor
SP	surfactant protein
SREBP	sterol regulatory element binding protein
TLR	toll-like receptor
TNF	tumor necrosis factor

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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