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REVIEW

Adoptive T-cell therapy for fungal infections in haematology patients

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The prolonged immune deficiency resulting from haematopoietic stem cell transplant and chemotherapy predisposes to a high risk of invasive fungal infections. Despite the recent advances in molecular diagnostic testing, early initiation of pre-emptive antifungal therapy and the use of combination pharmacotherapy, mortality from invasive mould infections remain high among recipients of allogeneic stem cell transplant. The increasing incidences of previously rare and drug-resistant strains of fungi present a further clinical challenge. Therefore, there is a need for novel strategies to combat fungal infections in the immunocompromised. Adoptive therapy using *in vitro*-expanded fungus-specific CD4 cells of the Th-1 type has shown clinical efficacy in murine studies and in a small human clinical study. Several techniques for the isolation and expansion of fungus-specific T cells have been successfully applied. Here we discuss the incidence and changing patterns of invasive fungal diseases, clinical evidence supporting the role of T cells in fungal immunity, methods to expand fungus-specific T cells in the laboratory and considerations surrounding the use of T cells for fungal immunotherapy.
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EPIDEMIOLOGY OF INVASIVE FUNGAL DISEASES IN HAEMATOLOGICAL MALIGNANCIES

Haematopoietic stem cell transplant

A large prospective surveillance study of 16 200 adult and paediatric haematopoietic stem cell transplant (HSCT) recipients over 2001 to 2006 in the United States reported the overall 1-year cumulative incidence of invasive fungal diseases (IFDs) in allogeneic HSCT recipients was 3.4% but reached 8.1% and 7.7% in patients undergoing transplantation from human leukocyte antigen (HLA)-mismatched-related and matched-unrelated donors respectively.[1] In autologous transplant recipients, the rate was 1.2%.[1] The incidence of IFD among adult and paediatric HSCT patients in China is higher, reported at 8.9% after allogeneic and 4.0% after autologous transplantation.[2] The cumulative incidences at 6 months in recipients of haploidentical and unrelated HSCT were 13.2% and 12.8%, respectively, compared with 4.3% in patients who received HLA-matched HSCT.[2]

Analyses of the distribution of IFDs showed that invasive aspergillosis is most common, accounting for 40–70% of infections, followed by invasive candidiasis (~25%), zygomycosis (~8%) and other moulds (~7%).[1–3] In paediatric HSCT patients however, *Candida* is the predominant fungal pathogen, responsible for 51% of IFDs compared with *Aspergillus* (26%).[4] The 1-year mortality associated with IFDs is ~75% in HSCT patients with invasive aspergillosis, ~65% in patients with invasive candidiasis, ~70% in patients with invasive zygomycoses and over 90% in patients with invasive fusariosis.[1]

Various risk factors contribute to the high incidence of IFDs in allogeneic HSCT recipients. These include severe neutropenia, lymphopenia, HLA disparity between the donor and recipient, graft-versus-host disease, the use of corticosteroid and immunosuppressive therapy, and diabetes.[2,3,5,6] Following allogeneic HSCT, full immune recovery can take up to a year. Innate immunity, including neutrophils and phagocytes, typically recovers within weeks after grafting.[7] However, recovery of adaptive immune components take longer, for example, B cells and CD8 T cells can take months to recover.[7] CD4 T-cell counts may be low for months to years and recovery is prolonged in older patients with poor thymic function and in patients receiving prophylaxis or treatment for graft-versus-host disease.[7] The reason for the lower incidence of IFDs in autologous HSCT is not entirely clear but is likely attributable to lower intensity conditioning, a shorter period of neutropenia and the absence of HLA disparity and graft-versus-host disease, and the consequent absence of mandatory immunosuppressive medication.[8]

Acute leukaemia

A large-scale retrospective study of >11 000 patients with haematological malignancy in Italy between 1999–2003 reported an overall IFD rate of 4.6%, with incidence rates of 12% in acute myeloid leukaemia and 6.5% in acute lymphoblastic leukaemia.[9]

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Invasive aspergillosis is the most common form, accounting for over 50% of all IFDs in acute leukaemia patients.[9] The percentage of patients with invasive aspergillosis who die from fungal disease has fallen over the last two decades, largely as a result of better diagnosis and the early initiation and use of improved fungal pharmacotherapy.

In patients with acute leukaemia, neutropenia, quantitative and qualitative alterations in monocytes and tissue macrophages, the use of broad-spectrum antibiotics, renal insufficiency, prior fungal infection and anti-fungal therapy, and active haematological disease leading to suppression of immune function are important risk factors for the development of IFDs.[10] In addition, colonisation of fungi in the gastrointestinal mucosa following acute mucosal damage caused by cytotoxic drugs is a risk factor in the pathogenesis of yeast-related IFDs.

Common fungal pathogens, treatment and changing patterns of IFDs in haematology patients

A number of studies have looked at the distribution of fungal isolates observed in clinical specimens obtained from recipients of HSCT.[1,3,11] *Aspergillus fumigatus* was the most common fungal pathogen in both autologous and allogeneic transplantation settings. Other positively identified *Aspergillus* species included *A. terreus*, *A. niger* and *A. flavus*. Among *Candida* infections, *C. albicans* and *C. glabrata* were most common, followed by *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. lusiteniae*. The *Mucor*, *Rhizopus* and *Absidia* species were common agents of zygomycoses. The less common fungal pathogens included *Fusarium*, *Scedosporium*, *Pneumocystosis*, *Cryptococcus*, *Alternaria*, *Exophiala* and the *Paecilomyces* species. Notably, co-infection by multiple fungal species is common in recipients of HSCT,[11] making treatment and management of IFDs challenging.

Amphotericin B was the mainstay of the treatment of invasive fungal infections until the mid-1990s. It has been replaced in the past two decades by more effective and less toxic drugs such as the less nephrotoxic lipid formulations of Amphotericin B, the broad spectrum triazoles (voriconazole, itraconazole, fluconazole and posaconazole), the echinocandins (caspofungin and micafungin) and the pyrimidine analogues (flucytosine). Voriconazole, posaconazole, caspofungin and lipid formulations of Amphotericin B are the common choices for treatment and prophylaxis of IFDs in haematology patients, also being administered empirically to patients with febrile neutropenia persisting 3–7 days after treatment with broad-spectrum antibacterials. Advances in molecular diagnostic testing and detection of the serum biomarkers β-glucan and galactomannan have facilitated prompt, targeted treatment and early initiation of pre-emptive therapy.[12] The selection of antifungal drug depends on the type, site and severity of fungal infection, potential for organ toxicity and possible interaction with other drugs. Caspofungin is the drug of choice for treatment of invasive candidiasis, voriconazole for invasive aspergillosis and lipid formulation of amphotericin B for zygomycosis; however, combination therapy using two or more of these agents is increasingly common.[3] Although these antifungal drugs are mostly effective in the treatment and management of IFDs, the cost associated with their use remains high. A more serious concern associated with the use of antifungals is the emergence of drug resistance. The overall resistance of *Candida* species to fluconazole and voriconazole is reported at around 3–6%.[13] Of notable mention is the increase of fluconazole resistance in *C. glabrata* from 7% in 2001 to 12% in 2004 as shown by data from the ARTEMIS Global Antifungal Surveillance Program.[14] Triazole resistance in *A. fumigatus* is increasingly being recognised, with resistance now reported in up to 6% of clinical isolates in the United States, United Kingdom and the Netherlands.[15–17] Although there has been no significant epidemiological shift in the susceptibility of *Candida* species to echinocandins, a case of breakthrough infections occurring after echinocandin therapy in an allogeneic HSCT recipient has been reported.[18] The prevalence of flucytosine resistance in yeast is below 2%; however, the high risk of yeast developing resistance to flucytosine has prompted its use in combination with other antifungal therapy, in particular Amphotericin B.

A number of mechanisms contributing to drug resistance in fungi have been proposed, for example, the induction of efflux pumps encoded by the multidrug-resistant or *Candida* drug-resistant genes, point mutations in genes encoding *ERG11* or *FKS* in *Candida* and *Cyp51A* point mutations in *Aspergillus*.[15,19] Although overall the incidence of antifungal resistance is low, it remains a serious problem in the management of high-risk haematology patients, particularly in those receiving a second transplant or having received prior antifungal therapy. The agricultural use of azole fungicides has also ignited concerns about the possibility of induction of resistant strains of fungi.[20] The emergence of previously less common IFDs in the haematology setting, for example, *C. krusei*, *C. glabrata*, zygomycetes and mucormycetes presents further clinical challenge for the treatment and management of IFDs.

ANTIFUNGAL IMMUNITY

Innate immunity

The innate immune system forms the first line of defence against fungal infection and serves two main functions (1) to mediate direct fungal destruction through phagocytosis or production of fungicidal molecules and (2) to orchestrate adaptive immune response through cytokine production, antigen presentation and T-cell stimulation. The cells with well-defined roles in fungal innate immunity include monocytes, macrophages, neutrophils and immature dendritic cells at the lining of the mucosal surface. Recognition of fungi is by pattern recognition receptors (PRRs), such as Toll-like receptors (for example, TLR2, TLR4 and TLR9), C-type lectin receptors (for example, Dectin-1, Dectin-2, DC-SIGN and mannose receptor) and galectin family proteins (for example, Galectin 3) expressed by the host cells. These receptors recognise fungal pathogen associated molecular patterns (PAMPs), which include zymosan, phospholipomannan, *O*-linked- and *N*-linked-mannans, β- and α-glucans, chitin and β-mannosides. The expression of these PAMPs by various fungal morphotypes and the corresponding PRR activated by them has been reviewed in depth by Romani.[21] The engagement of PRRs on innate cells activates signalling cascades, such as the MyD88-, Syk- and Ras-dependent pathways leading to phagocytosis and the production of defensins, chemokines, cytokines and reactive oxygen species. Dendritic cells are unique in their capacity to decode fungal information, migrate to the local lymph nodes and activate adaptive fungal responses, both by production of immunomodulatory cytokines (such as type-1 interferon (IFN), interleukin (IL)-4, IL-6, IL-10, IL-12 and IL-23) and direct stimulation of T cells. Specific single-nucleotide polymorphisms occurring in cytokine, cytokine receptor or PRR genes which alter components of the innate fungal immune response and thereby increase susceptibility or resistance to fungal infections have been reported.[21] Clinically, neutropenia and defects in the formation of reactive oxygen species (as observed in chronic granulomatous disease) are well-recognised factors for the development of IFDs.[22,23] In recent years, natural killer (NK) cells have received a lot of interest for their potential role in innate fungal immunity. Although the exact mechanisms by which NK cells recognise fungus or fungus-infected cells are not completely understood, direct effects of NK cell-derived

perforin and IFNγ on fungal hyphal death have been observed.[24,25] Furthermore, early initiation of NK activity is protective in neutropenic mice with invasive aspergillosis.[26,27]

Adaptive immunity

Similar to innate immunity, fungus-specific adaptive immunity also serves two main functions (1) act in concert with innate immunity to clear fungal infection and (2) induce long-term immunological memory in the event of a recall infection by the same or similar pathogen. The role for CD4 T cells in fungus-specific adaptive immunity is now well established and specific responses by all CD4 T-cell subsets (T helper (Th)-1, Th2, Th17 and regulatory T cells) have been observed. The type of response is orchestrated by the milieu of cytokines produced by innate cells, for example, production of IL-12 by dendritic cells results in a predominant Th1 response, IL-23 and IL-6 result in a Th17 response, IL-4 results in a Th2 response and IL-10 in a regulatory T cell response. Protective immunity, correlating with production of defensins, inflammation, neutrophil recruitment and fungal clearance is observed following activation of Th1 and Th17 cells. In contrast, the activation of Th2 and regulatory T cells corresponds to inhibition of fungal clearance and immunosuppression, respectively.

Th1 response leading to production of IFNγ, tumour necrosis factor (TNF)-α and IL-2 is very well characterised and there is a strong body of evidence suggesting that defects in Th1 numbers and cytokine response correlate with higher fungal burden.[28–30] The production of Th1 cytokines by T cells is mediated by direct contact with activated dendritic cells, as the use of a major histocompatibility complex class II blocking antibody abrogates the Th1-specific response to fungi.[31] Th1 cells also form the basis of immunological memory to specific fungal infections[32] and *A. fumigatus*-specific T cells can be detected in low frequency in the peripheral blood of healthy individuals.[33] Further evidence supporting the antifungal role of Th1 cells is discussed in the section 'Clinical evidence supporting the role of T cells' below. A protective role of Th17 cells in fungal immunity, in particular to *Candida* infections, has also been observed.[34,35] While the contribution of CD4 T cells in fungal adaptive immunity is very well characterised, recent studies have reported that $CD8^+$ T cells can also mediate protective immunity against *Aspergillus* and *Candida* infections.[36,37]

IMMUNOTHERAPY FOR FUNGAL INFECTIONS

In a recent review, Armstrong-James and Harrison discussed various immunotherapy options for fungal infections.[38] These include the use of IFNγ therapy for chronic granulomatous disease, cryptococcal meningitis and transplant-associated fungal disease; antibody therapies using 18B7 (targeting *Cryptococcus neoformans* capsular polysaccharide) for cryptococcal meningitis and Mycograb (targeting *Candida* heat-shock protein-90) for invasive candidiasis; adoptive cell therapies using antigen-specific T cells and antigen-pulsed dendritic cells for invasive aspergillosis and/or candidiasis; granulocyte transfusions for neutropenic sepsis; and gene therapy (targeting the mutated nicotinamide adenine dinucleotide phosphate oxidase gp91 subunit) for chronic granulomatous disease. Although many of these have shown promising results in pre-clinical studies, only IFNγ therapy has proceeded to the clinical main stage, now being approved for use in patients with chronic granulomatous disease.[38]

Clinical observations supporting the role of T cells in fungal immunity

The identification of the adaptive immune system as a crucial component of host antifungal immunity did not surface till the early 2000s when lymphopenia was first identified as an important risk factor for the development of IFDs. This was prompted by studies reporting a high incidence of late-onset infections (occurring after the period of neutrophil recovery) in HSCT recipients and a high incidence of fungal infections in non-neutropenic HIV-infected patients. Recent data from the Transplant-Associated Infection Surveillance Network database from 23 transplant centres across the United States was consistent with those findings, reporting the median time from transplantation to the onset of candidiasis, aspergillosis, fusariosis and zygomycosis as 61, 99, 123 and 135 days, respectively,[1] correlating with the period of T-cell immunodeficiency. As previously highlighted, Th1 type CD4 T cells have a crucial role in fungus-specific immunity.

The roles for the Th1 cytokines IFNγ and TNFα in protecting against fungal infections have been demonstrated through the use of neutralising antibodies in murine models of invasive aspergillosis.[29,30] These observations are consistent with clinical findings. For example, the increased secretion of IFNγ by lymphocytes in response to stimulation with *Aspergillus* antigens correlates with a better outcome in transplant patients with invasive aspergillosis.[39] Conversely, the administration of TNFα inhibitors to patients for the treatment of inflammatory diseases such as rheumatoid arthritis increases the incidence of fungal infections.[40] The specific roles for these cytokines in fungal response is now well understood; TNFα increases the oxidative killing of *Aspergillus* hyphae by neutrophils, enhances the phagocytic capacity of alveolar macrophages and promotes neutrophil accumulation in the lungs of *Aspergillus*-infected animals.[30,41] IFNγ promotes increased phagocytosis, nitric oxide production and fungal killing by macrophages, and skews the fungal response towards a protective Th-1 type.[42]

In HSCT patients, *A. fumigatus*-specific T-cell immunity is defective for up to a year following transplantation, correlating with the period when patients are at highest risk of infection.[33,43] In a recent study by Jolink *et al.*, *A. fumigatus* Crf1 and Catalase-1-specific $CD4^+$ T cells were detected in the blood of patients recovering from invasive aspergillosis, but not in patients with progressive disease.[44] Consistent with the observations in transplant patients, an inverse correlation between $CD4^+$ T-cell numbers and the incidence of IFDs has been observed in HIV-infected patients.[28] We have shown that *A. fumigatus*-specific T cells, which have been expanded *in vitro*, predominantly produce Th1 cytokines, in particular TNFα, but also IFNγ, when restimulated.[31] Specific Th1 cell responses against *Fusarium*[45] and *Candida* have been demonstrated by others.[46–48]

T-CELL THERAPY FOR FUNGAL INFECTIONS

Murine studies

The first *in vivo* evidence for the importance of the adaptive immune system in dealing with fungal infections came from studies performed by *Cenci et al.*[49] Multiple intranasal inoculations of *A. fumigatus* conidia in immunocompetent mice caused a transient, self-limiting infection that conferred resistance to subsequent infection,[49] suggesting induction of immunological memory after the first fungal encounter. In later studies, the treatment of mice with a culture filtrate of *Aspergillus* protected against subsequent intranasal or intravenous infection with live fungal conidia.[32] Improved survival in treated mice correlated with increased recruitment of lymphocytes, macrophages and neutrophils to the site of infection and reduced local and systemic

fungal burden. Higher levels of IFNγ and IL-2 in the culture supernatants of activated lung cells were observed in treated mice. Splenic $CD4^+$ T cells from treated mice showed strong proliferative response to restimulation with antigen *in vitro* and upon adoptive transfer, prolonged the survival of unimmunised mice subsequently infected with a lethal dose of *A. fumigatus*.[32] This study was the first to provide evidence that *Aspergillus*-specific immunity could adoptively be transferred from one host to another, suggesting a core role for cells with immunological memory in this protective response. In subsequent studies, Bozza *et al.*[51] demonstrated an intermediary role for dendritic cells in the $CD4^+$ T-cell response to *A. fumigatus*. Splenic dendritic cells transfected with conidial RNA of *A. fumigatus* induced proliferation of and IFNγ production by allogeneic $CD4^+$ T cells *in vitro*. In an adoptive T-cell transfer model, the infusion of allogeneic conidia-pulsed or conidial RNA-pulsed dendritic cells protected mice otherwise susceptible to aspergillosis against infection-induced death. This protection correlated with *in vivo* production of IFNγ by $CD4^+$ T cells. Subsequent experiments in mice receiving T-cell-depleted allogeneic stem cell grafts showed that infusion of *Aspergillus* conidia or conidial RNA pulsed donor-derived dendritic cells before infection increased resistance to infection, restricted fungal growth and prevented disease dissemination. The use of *Apsergillus*-specific $CD4^+$ T cells isolated from the spleens of immunised mice which were restimulated *in vitro* were also protective, extended the life of mice and were associated with accelerated recovery of myeloid and lymphoid compartments. These studies taken together support the notion that fungus-specific immunity in an immunocompetent host is induced through fungal exposure. Most importantly, this immunity can be adoptively transferred through use of fungus-antigen pulsed dendritic cells or fungus-specific T cells to an immunocompromised host.

Human studies

There is only one reported study to date which has assessed the safety and efficacy of adoptively transferred fungus-specific T cells in humans. This was performed by Perruccio *et al.* in patients undergoing haploidentical stem cell transplant with rigorously T-cell-depleted grafts.[43] Peripheral blood mononuclear cells from healthy donors were stimulated with irradiated donor-derived antigen-presenting cells, which had been pulsed with heat-treated conidia of *Aspergillus*. $CD4^+$ T cells were cloned by limiting dilution and expanded with the support of donor-derived feeder cells and IL-2. Ten haploidentical stem cell transplant recipients with evidence of invasive aspergillosis received a single infusion of 1×10^5–1×10^6 cells per kg of expanded donor-derived anti-*Aspergillus* T-cell clones a median of 21 days post transplantation. *Aspergillus*-specific $CD4^+$ T cells were detected within 3 weeks of infusion and 9 of 10 patients cleared the infection within 7.8 ± 3.4 weeks. In all treated patients, fungal galactomannan levels measured between 6 and 12 weeks of infusion declined to below $1\,ng\,ml^{-1}$. In contrast, in the matched control cohort, who did not receive adoptive T-cell therapy, 6 of 13 patients succumbed to infection within 4.8 ± 1.2 weeks of diagnosis and *Aspergillus* galactomannan levels remained elevated for the duration of the study. In addition, $CD4^+$ T-cell clones could only be detected in very low frequencies 9 to 12 months after transplantation in this cohort. Although this was only a small study, it has provided proof-of-principle data for the clinical efficacy of *in vitro* expanded *Aspergillus* specific T cells in humans.

GENERATION OF FUNGUS-SPECIFIC T CELLS FOR CLINICAL CELL THERAPY

Since the demonstration of clinical efficacy of *A. fumigatus* specific T cells in humans, a number of groups have published methods to expand fungus-specific T cells *in vitro*. These studies have focused on the following: (1) identifying a suitable antigen source to use for specific stimulation of fungus-specific cells and (2) identifying a reliable cell culture method to expand fungus-specific T cells. Results from some of these studies with promising outcomes are summarised in Table 1.

Antigens

Very little is known about specific epitopes in fungi that induce an antifungal T-cell response. In a recent study, Bacher *et al.* determined the immunogenic capacity of selected proteins in *A. fumigatus* by analysing activation-dependent expression of CD154 by flow cytometry.[51] The stimulation of healthy donor blood mononuclear cells with overlapping peptide mixes or recombinant proteins, such as Aspf1, Aspf16, Aspf22, Catalase-1, Gel1, Crf1, Crf2 and Pmp20 proteins induced specific CD154 and TNFα co-expression. Of considerable interest was the finding that the T-cell responses to single *A. fumigatus* proteins represented only a small fraction of the total T-cell response observed against the crude lysate, confirming the presence of a number of distinct immunodominant antigens. An analysis of 22 patients with invasive aspergillosis showed that positive T-cell response towards two or more of the *Aspergillus* antigens Pep1, Crf1, Gel1, Sod1, α1-3 glucan and β1-3 glucan correlated with a favourable disease outcome, indicating the presence of protective immunogenic epitopes within these fungal proteins.[52] In contrast, patients with a complete absence of IFNγ response or a response to only one antigen showed poor disease outcome. In a recent study, Jolink *et al.* identified five novel Crf1 epitopes and 30 novel Catalase-1 epitopes restricted to specific HLA-DR alleles.[53] Despite all of these studies, only one specific epitope has to date reliably been used to expand fungus-specific T cells *in vitro*. This is the p41 peptide encoded within the *Aspergillus* Crf1 protein. This epitope, identified by Stuehler *et al.*, was shown to be restricted to the HLA-DRB1-03, -04 and -13 alleles, and could successfully be used to expand *A. fumigatus*-specific T cells from donors with the appropriate HLA alleles.[47] Expanded cells demonstrated cross-reactivity with *C. albicans*, owing to epitope sharing between these fungi.

The previously published murine vaccination and disease prevention studies have used live or heat-treated conidia and conidial RNA.[32,43,49] In the human study, Perruccio *et al.* used heat-treated conidia for the expansion of *Aspergillus*-specific T-cell clones.[43] Results from all these studies have been promising, but in the current regulatory environment many such antigen sources will not be approved for manufacturing of cells for clinical use. In 2004, Braedel *et al.* described a cellular extract (lysate) of the germinated spores from a clinical isolate of *A. fumigatus* lysed in Tris-HCl.[54] This lysate showed potent lymphoproliferative activity and was successfully used to culture *Aspergillus*-specific T cells.[55] We adapted the method of Braedel *et al.*[54] to produce a water-soluble lysate free from acid or adjuvants from the germinated conidia of an environmental strain of *A. fumigatus* and used it to culture *Aspergillus*-specific T cells for clinical use. This lysate displayed lymphoproliferative activity *in vitro*,[31] similar to that observed using an acid-based lysate.[54] The advantage of using such an antigen source is the representation of the majority of antigenic epitopes and relevance for a range of HLA types, which would be absent if an HLA-specific epitope is used. In a more recent

Table 1 Reported methods for *A. fumigatus* T-cell isolation and expansion *in vitro*

Target pathogen(s)	*Antigen source*	*Method*	*Cell numbers (initial/final); yield*	*Total time to generate T cells (days)*	*CD4/CD8 ratio; % antigen specific CD4 T cells*	*Reference*
A. fumigatus	Water-soluble lysate	IFNγ capture-based isolation, stimulation with antigen-pulsed APC, feeder cells and IL-2	$1.1\times10^9/2\times10^7$; 1.8%	14	87/3; 10.3[a]	Tramsen *et al.*[57]
A. fumigatus	Crf1/p41 peptide	CD154 bead-based isolation, culture with feeder cells, IL-2, IL-7 and IL-15	$1\times10^7/3.5\times10^7$; 350%	14	ND; 50.6[b]	Stuehler *et al.*[47]
A. fumigatus	Crf1/p41 peptide	CD154 bead-based isolation, culture with feeder cells, IL-2, IL-7 and IL-15	$6.86\times10^7/1.86\times10^7$; 27%	15	ND; 40[b]	Khanna *et al.*[58]
A. fumigatus	Water-soluble lysate	Stimulation with antigen-pulsed APC, culture with IL-2, IL-7 and IL-15	$7.4\times10^6/1.94\times10^8$; 263%	21	94/4.5; 10.9[a]	Gaundar *et al.*[31]
A. fumigatus, C. albicans, R. oryzae	Water-soluble lysate	IFNγ capture based isolation, stimulation with antigen-pulsed APC, feeder cells and IL-2	$1.1\times10^9/5.4\times10^7$; 4.7%	13	98.5/0.4; 13.3[a]	Tramsen *et al.*[46]

Abbreviations: APC, antigen-presenting cells; ND, not determined.
[a]Indicated by expression of TNFα following restimulation with antigen pulsed APC.
[b]Indicated by staining with p41 tetramer antibody.

study, a combination of overlapping peptide mixes from Crf1, Gel1 and Pmp20 proteins of *Aspergillus* was shown to induce a T-cell response to almost similar levels to that observed with the lysate, raising the possibility of using overlapping pepmixes from known immunogenic proteins to generate cells for clinical use.[56]

Isolation and expansion of fungus-specific T cells

Various methods to expand fungus-specific T cells *in vitro* have been assessed. In studies by Beck *et al.*[55] and Tramsen *et al.*[57], an immunobead capture method based on activation-dependent production of IFNγ was used to isolate *Aspergillus*-specific cells following stimulation of bulk peripheral blood mononuclear cells with lysate; isolated cells were expanded *in vitro* with the support of autologous feeder cells, further stimulation with autologous dendritic cells pulsed with antigen, and IL-2. More recently, Tramsen *et al.* applied the same method to expand T cells with specificity against *Aspergillus*, *Candida* and *Fusarium* in a single culture using a combination of lysates from these fungi.[46] In 2011, Khanna *et al.* described an immunobead selection based on activation-dependent expression of CD154 to isolate fungus-specific T cells; isolated cells were cultured with autologous feeder cells, IL-2, IL-7 and IL-15.[58] In our study, we used two stimulations of bulk peripheral blood mononuclear cells using autologous dendritic cells pulsed with *A. fumigatus* lysate, then expanded the cells with a cocktail of IL-2, IL-7 and IL-15.[31] Despite differences in the isolation and expansion of fungus-specific T cells, all of these methods have generated numbers that can be scaled for clinical use. The clinical utilisation of each of these methods will depend on the starting cell number required (for example, studies by Beck *et al.*[55] and Tramsen *et al.*[57] required large starting cell numbers, which can perhaps only be achieved using a leukapheresis product or a stem cell collection), the availability of clinical grade reagents, the cost of manufacturing and the feasibility of the procedure for routine clinical cell production.

Genetic manipulation to confer direct antifungal activity to T cells

Antigen-specific T cells can induce direct cytotoxic activity against virus, for example cytomegalovirus-infected cells. However, this is not clearly evident in the case of fungal infections and as previously discussed, the majority of responses observed against fungi both *in vitro* and *in vivo* are CD4 T-helper cell mediated. Kumaresan *et al.* recently described genetic modification of T cells to express a chimeric antigen receptor encoding the fungal PRR Dectin-1 in association with T-cell receptor signalling components.[59] Cells expressing Dectin-1 could be enriched through stimulation with antigen-presenting cells coated with Dectin-1 agonist and expanded *in vitro* with the support of IL-2 and IL-21. Cultured cells demonstrated direct antifungal activity *in vitro* and showed efficacy in murine studies.

IMPORTANT CONSIDERATIONS FOR THE USE OF T CELLS FOR FUNGAL IMMUNOTHERAPY

It is clear that fungus-specific T cells can reliably be generated *in vitro*. However, certain factors will be crucial when considering the use of T-cell immunotherapy for fungal infections. Selection of the patient and timing of T-cell infusion will undoubtedly be paramount for the success of T-cell therapy. These are discussed below.

There are toxicity concerns associated with use of T cells to treat active infection, these include the overstimulation of antigen-specific T cells that could potentially result in cytokine release syndrome and tissue damage. Although Perruccio *et al.* reported no such adverse effects,[43] it is conceivable that the site and extent of systemic infection will be an important factor when assessing suitability of the patient for

T-cell therapy. Genetically modifying T cells to include an inducible suicide gene that can electively be activated in the event of toxicity, such as that explored in cancer immunotherapy,[60–62] will be useful to address toxicity concerns.

Timing of T-cell infusion may be critical and it is likely that infusion of T cells at a time when patients are at highest risk of infection, i.e. within the first 3 months after HSCT or after conclusion of immunosuppressive therapy will be most beneficial. In a recent study, Tramsen *et al.* showed that commonly used immunosuppressive drugs have adverse effects on proliferation and cytokine production by *Aspergillus*-specific T cells.[63] These data suggest that adoptive immunotherapy using fungus-specific T cells may be best used in the context of non-pharmacological graft-versus-host disease strategies such as $CD34^+$ selection or post-transplant cyclophosphamide. In addition, infusion of T cells may be most effective in patients who have achieved complete or partial recovery of their innate immune system. Methods to bypass requirement of the innate immune system, for example, through the use of genetically engineered T cells, such as Dectin-1-expressing T cells as recently demonstrated in murine models,[59] may allow fungus-specific T-cell therapy to be more rapidly administered. This latter strategy however is still in early developmental phases and will require intensive pre-clinical testing before it is deemed safe for human use.

CONCLUSION

Profound systemic (in particular cellular) immune suppression resulting from HSCT and intensive chemotherapy predisposes patients to potentially fatal IFDs. Despite advances in diagnostic testing, the introduction of azoles and the use of the best available prophylactic and empirical anti-fungal pharmacotherapy, immunosuppressed haematology patients continue to die from fungal-infection-related diseases. Just as resolution of neutropenia is the key to minimising risk from bacterial sepsis, improvements in cellular immunity are key to reducing risk from fungal infection and improving IFD outcomes. Data in literature support the role of Th1 cells in fungal adaptive immunity. Adoptive transfer of fungus-primed T cells isolated from immunised mice has clinical efficacy in murine models of invasive aspergillosis. Allogeneic donor-derived fungus-specific T-cell clones have also shown clinical efficacy in a small cohort of haploidentical stem cell transplant patients. Various methods to expand *A. fumigatus*-specific T cells in the laboratory have now been described and can be scaled to produce clinical grade T cells. Whether the use of fungus-directed T-cell therapy will improve patient outcomes and reduce the need for fungal pharmacotherapy will not be known until larger-scale clinical trials are conducted. It is possible that the benefits of cell therapy for fungal prophylaxis and treatment may parallel those seen for viral infections such as cytomegalovirus, Epstein–Barr virus and adenovirus.[64–69] Specific T-cell therapy may be a novel therapeutic option for a group of patients for whom current treatment options are at best suboptimal.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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