Generation and Characterization of Anti-
Candida T Cells as Potential Immunotherapy in Patients with Candida Infection after Allogeneic Hematopoietic Stem-Cell Transplant

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Because lymphocytes play a major role in the host response to Candida infection, adoptive transfer of anti-Candida T cells might be a therapeutic option in patients undergoing allogeneic hematopoietic stem-cell transplant (alloHSCT) who have invasive Candida infection. Using the interferon (IFN)–γ secretion assay, we isolated human anti-Candida T cells after stimulation with a cellular extract of C. albicans. These cells were expanded within 4 weeks to an average number of T helper 1 type lymphocytes and significantly lost their alloreactive potential, compared with the original cell population. The generated cells were also stimulated by antigens of C. tropicalis but not by antigens of C. glabrata or various molds. In addition, generated anti-Candida T cells were able to induce damage to C. albicans hyphae and significantly increased hyphal damage induced by human neutrophils. Our data suggest that the generation of functionally active anti-Candida T cells is feasible and may be a promising treatment option for patients undergoing alloHSCT.

Patients undergoing allogeneic hematopoietic stem-cell transplant (alloHSCT) are at high risk of life-threatening invasive fungal infections (IFIs) [1]. Although the majority of IFIs are caused by molds [2], the incidence and mortality of invasive yeast infections are still unacceptably high. Most yeast infections are caused by Candida species, in particular by C. albicans, but the incidence of nonalbicans species, such as C. glabrata and C. krusei, is increasing [1]. Candida species may induce a broad variety of infections, ranging from non-life-threatening mucocutaneous illness to invasive disease involving virtually any organ. A recent analysis reported an associated mortality rate of 33% for Candida infections, which, despite the availability of new antymycotics, was not an improvement over that of previous studies [3]. Preliminary results of therapeutic approaches such as granulocyte transfusions and interferon (IFN)–γ have suggested a benefit for outcome but have yet to be validated [4, 5]. By contrast, there is a growing body of evidence that the adaptive immune system has a major impact on the host response to Candida infection [6, 7]. For example, dendritic cells pulsed with fungal RNA induce protective immunity to C. albicans after HSCT in mice [8]. Because IFN-γ–producing Th1 cells play a crucial role in the control of IFIs, the reconstitution of adaptive cell immunity with antigen-specific T cells is a promising approach in patients undergoing alloHSCT [6, 7]. This strategy has been well established for patients with cytomegalovirus (CMV) disease or with Epstein-Barr virus...
(EBV–associated posttransplant lymphoproliferative disease [9, 10], and preliminary results of transferring a functional immune response to *Aspergillus* species after alloHSCT are promising [11]. By contrast, data regarding donor-derived *Candida*-specific human T cells are missing. In the present study, we developed a simple and feasible strategy for the generation of functionally active anti-*Candida* T cells, which might become a treatment option in patients with invasive *Candida* infection after alloHSCT.

**SUBJECTS, MATERIALS, AND METHODS**

**Study subjects.** After informed consent was provided, 150 mL of blood was obtained from healthy adult volunteers for the isolation of anti-*Candida* T cells and the preparation of monocyte-derived autologous antigen-presenting cells (APCs). None of the individuals had a history of IFI or atopic disease. The protocol was approved by the local ethics committee.

**Fungal antigens.** The water-soluble extract of *C. albicans* (CA444), grown on yeast extract peptone dextrose (YPD), was prepared as described elsewhere [12]. Antigens were determined to be endotoxin free using the limulus amebocyte lysate assay (BioWhittaker) and were kept in aliquots at −80°C until use. For testing specificity, antigens of various yeasts and molds, grown on YPD medium and on Sabouraud agar plates respectively, were prepared identically.

**Generation and expansion of purified anti-*Candida* T cells.**

Stimulation of peripheral blood mononuclear cells (PBMCs) and enrichment of cytokine-secreting cells using the cytokine secretion assay (CSA; Miltenyi Biotech) was performed as described elsewhere [13, 14] (figure 1). In brief, 1 × 10⁷ PBMCs obtained by Ficoll-Paque (Biochrome) density-gradient centrifugation from 100 mL of blood were adjusted to a final concentration of 1 × 10⁶ cells/mL in cytotoxic T lymphocyte (CTL) medium that contained RPMI 1640, 100 IU/mL penicillin G, 100 μg/mL streptomycin (Invitrogen), and 10% pooled heat-inactivated human serum (HS). After 16 h of coincubation with 7.5 μg/mL *C. albicans* antigens, stimulated cells were selected using the IFN-γ CSA MiniMACS device (Miltenyi Biotech). Isolated cells were placed on a 24-well plate (Nunc) in 2 mL of CTL medium that contained 50 IU/mL recombinant human interleukin-2 (rhIL-2; Chiron) and 5 × 10⁶ irradiated LCLs, and 30 ng/mL OKT-3. Cells were cultured for 14 days, supplemented with 50 IU/mL rhIL-2 on days 1, 4, 7, and 10 and with fresh medium when necessary (figure 1).

**Figure 1.** Schematic diagram for generation and characterization of anti-*Candida* T cells. After stimulation of 1 × 10⁶ peripheral blood mononuclear cells (PBMCs) with cellular extracts of *C. albicans*, cytokine-producing cells were isolated using the cytokine secretion assay. Cells were expanded for up to 14 days and were purified by limiting dilution assay. After further expansion, cells were characterized by phenotyping, intracellular cytokine staining, and T cell receptor (TCR) spectratyping and were assessed for expansion on restimulation, alloreactivity, and anti-*Candida* activity.

Characterization of phenotype and intracellular cytokine staining. Phenotyping of purified anti-*Candida* T cells was performed by 4-color flow cytometry (XL-MCL; Beckman Coulter) using monoclonal antibodies (MAbs) against CD3, CD4, CD8, CD14, CD19, CD45RA, CD45RO, CD56, T cell receptor (TCR)–α/β, TCRγ/δ, and HLA-DR labeled with fluoroescin isothiocyanate (FITC), R-phycocerythrin (PE), PE–Texas red, or PE–cyanin. All antibodies were obtained from Coulter Immunotech.

Intracellular cytokine flow cytometry (ICC) was performed as described elsewhere [15]. In brief, autologous monocyte-derived APCs, obtained from PBMCs by the adherence method, were incubated overnight with *C. albicans* antigens (7.5 μg/mL). Then, the generated anti-*Candida* T cells were stimulated for 6 h with *Candida*-loaded APCs (effector:stimulator cell ratio, 5:1) and costimulated with MAbs against CD28 (BD Biosciences) and CD49d (Immunotech; 2 μg/mL each). For testing specificity, generated T cells were stimulated with an

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tigens of *C. albicans* (CA444 and SN-1-6946; Greer Laboratories), *C. glabrata* (CBS138), *C. tropicalis* (CBS94), *Aspergillus fumigatus* (CBS144.89), *Penicillium chrysogenum* (IP1652), and *Alternaria alternata* (IP1563) (7.5 μg/mL each). APCs with or without control antigen (tetanus toxoid; Chiron Behring) were used as negative controls. Positive controls were obtained by stimulation with 0.5 μg/mL PMA (Sigma) and 1 μg/mL ionomycin (Sigma). Brefeldin A (10 μg/mL; Sigma) was added for the last 5 h of incubation. Cells were permabilized using BD FACS Permeabilizing Solution 2 (BD Biosciences) and were stained with MAbs against CD3, CD8, IFN-γ, tumor necrosis factor (TNF)-α, IL-4, IL-10 (BD Biosciences), and CD4 (Dako Cytomation) labeled with FITC, PE, peridinin-chlorophyll-protein complex (PerCP), or allophycocyanin. Analyses were performed using the FACSCalibur flow cytometer (Becton Dickinson).

**Complementarity-determining region 3 (CDR3) spectratyping of TCR-Vβ transcripts.** Total RNA was prepared from 1 x 10⁵ PBMCs of the original fraction or of anti-*Candida* T cells using TRIzol reagent (Invitrogen). cDNA was synthesized by random priming using a cDNA synthesis kit (QuantiTect; Qiagen) and was subjected to TCR-Vβ gene family–specific polymerase chain reaction (PCR) in 26 separate reactions, each of which contained 1 of 26 Vβ families in combination with a universal Cβ-specific primer [16]. In a second step, runoff products were generated from each Vβ-specific PCR product by each of 13 fluorescence-labeled, Jβ-specific oligonucleotides [17]. Fragments were separated on an automated 48-capillary DNA sequencer (3730 DNA Analyzer; ABI). Length and fluorescence intensities of the CDR3 of the different Vβ-Jβ combinations were determined using GeneScan 500LIZ (Applied Biosystems) in accordance with standard methods and were analyzed using GeneMapper software (version 3.5; Applied Biosystems).

**Carboxy fluorescein diacetate succinimidyl ester (CFSE) staining.** CFSE (Molecular Probes) staining was performed as described elsewhere [15]. In brief, anti-*Candida* T cells and autologous PBMCs were labeled with 0.6125 μmol/L CFSE and washed 3 times with RPMI 1640 that contained 15% HS. Then, 1 x 10⁵ anti-*Candida* T cells were plated into each well of a flat-bottom 24-well plate. For testing proliferation on restimulation, irradiated autologous APCs (2 x 10⁵), either unstimulated or stimulated with *C. albicans* antigens, were added. For testing alloreactivity, irradiated allogeneic APCs (2 x 10⁵) were
Figure 3. Response of anti-Candida T cells to stimulation with various fungal antigen extracts. Shown is interferon (IFN)–\( \gamma \) staining of anti-Candida T cells stimulated with autologous antigen-presenting cells loaded with antigens of C. albicans, C. tropicalis, C. glabrata, Alternaria alternata, Penicillium chrysogenum, and Aspergillus fumigatus.

IFN-\( \gamma \) added to labeled PBMCs or to anti-Candida T cells. rhIL-2 (5 IU/mL) was supplemented on days 1 and 4. On days 0, 4, and 7, cells were stained with CD3 PE, CD8 PerCP (BD Biosciences), and CD4 allophycocyanin (Dako) and analyzed using flow cytometry (FACSCalibur).

**Cytokine assessment.** IFN-\( \gamma \) concentrations were measured in duplicate using a commercially available ELISA (BD Biosciences) with a detection limit of 4.7 pg/mL.

**Assessment of antifungal activity.** Assessment of fungal damage was performed using a blood culture isolate of C. albicans (Institute of Medical Microbiology, University of Frankfurt, Frankfurt, Germany) by means of the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxyanilide (XTT; Sigma) colorometric assay plus 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q0; Sigma), as described elsewhere [15, 18] with some modifications. Two colony-forming units of blastoconidia grown on Saboraud agar plates were washed in Hanks’ balanced salt solution with calcium and magnesium (Invitrogen). A total of 5 \( \times 10^4 \) conidia were transferred into each well of a 96-well flat-bottom plate (Becton Dickinson), suspended in RPMI 1640 that contained 10% HS, and incubated for 6 h at 37°C to allow germination of blastoconidia into hyphae. Then, APCs (5 \( \times 10^4 \) cells/well), purified T cells (2.5 \( \times 10^5 \) cells/well), and, after 1 h of incubation, polymorphonuclear leukocytes (PMNs; 5 \( \times 10^4 \) cells/well) were added in various combinations. Each condition was tested in triplicate.

Plates were incubated for another 4 h and washed twice, and 150 \( \mu \)L of XTT solution (PBS that contained 0.25 mg/mL XTT sodium salt and 40 \( \mu \)g/mL coenzyme Q) was added for 1 h. Then, 100 \( \mu \)L were transferred to a new plate. Antifungal activity was assessed spectrophotometrically at 450 nm using 690 nm as the reference and calculated according to the following formula [19]: percentage hyphal damage = \( (1 - X/C) \times 100 \), where \( X \) is the absorbance of experimental wells and \( C \) is the absorbance of control wells with hyphae only. For the assessment of antifungal activity, purified anti-Candida T cells of 3 different donors were tested 3 times each.

**Statistical analysis.** Data were analyzed using GraphPad Prism (version 4.00; GraphPad Software) and compared by 1-way analysis of variance followed by Dunnett’s correction for multiple comparisons. Two-sided \( P \leq .05 \) was considered to be statistically significant.

**RESULTS**

**Generation of anti-Candida T cells.** Anti-Candida T cells were enriched from 1 \( \times 10^8 \) PBMCs from 4 healthy volunteers by means of IFN-\( \gamma \) CSA. After stimulation with C. albicans antigens for 16 h, 6.2 \( \times 10^5 \) cells (range, 1.8-37.5 \( \times 10^5 \) cells), on average, were obtained, which were expanded during a culturing period of 12–14 days to a median number of 1.9 \( \times 10^7 \) cells (range, 1.0–2.4 \( \times 10^7 \) cells). The assessment of antigen-
triggered production of cytokines to determine the frequency and functionality of antigen-specific cells revealed a significant proportion of IFN-γ- and TNF-α-producing CD3⁺CD4⁺ T cells (median, 7.6% [range, 1.2%–19.7%] and 12.2% [range, 2.2%–27.6%], respectively).

To minimize possible contamination with small fractions of unspecific T cells, B cells, and NK cells, limiting dilution assay was performed, followed by another culturing period of 14 days. This resulted in an average number of $2.6 \times 10^5$ cells (range, 0.9–5.8 $\times 10^5$ cells) (6 different cell populations derived from 4 individuals). Flow cytometry revealed a highly homogenous population of CD3⁺CD4⁺ cells (median, 98.1% [range, 91.6%–99.3%]). In addition, the cells homogeneously expressed CD45RO, HLA-DR, and TCRα/β, indicating an activated memory Th cell population (data not shown). Functional assessment by ICC revealed that, after restimulation with C. albicans antigens, an average of 8.6% and 20.5% of these cells (range, 4.8%–58.2% and 5.8%–72.4%) secreted IFN-γ and TNF-α, respectively, which was significantly higher than the percentage of this cell population among negative controls ($<0.2\%$; $n = 6$) (figure 2). Only a marginal percentage of the generated cells secreted Th2 cytokines such as IL-4 or IL-10 ($<1\%$ each; data not shown). Importantly, IFN-γ production was detected in none of the settings in CD3⁺CD8⁺ T cells (data not shown). Cryopreservation in liquid nitrogen for up to 6 months did not affect anti-Candida T cells, given that their ability to expand and to produce IFN-γ on stimulation was comparable to that of freshly prepared anti-Candida T cells (data not shown).

**TCR spectratyping.** Similar to healthy individuals, a normal Gaussian distribution of the CDR3 of all Vβ-1β gene combinations of the TCR was observed in unselected CD4⁺ T cells (data not shown). After the selection of anti-Candida T cells, the TCR rearrangement became skewed. T cell clones changed from a polyclonal to an oligoclonal pattern. Although different Vβ and Iβ families could still be found in the composition of the TCR, the Gaussian distribution disappeared in nearly all of them. This restriction in the TCR repertoire was even stronger in the fraction of the highly purified anti-Candida T cells obtained after the limiting dilution assay was performed (data not shown).

**Specificity of anti-Candida T cell response.** The specificity of anti-Candida T cells was assessed by means of ICC using cellular extracts of various yeasts and molds. Although similar proportions of IFN-γ-producing CD3⁺CD4⁺ T cells were seen after stimulation with various antigens of C. albicans and C. tropicalis, no significant IFN-γ production was observed after stimulation with C. glabrata, Aspergillus fumigatus, P. chrysogenum, and Alternaria alternata (figure 3).

**Restimulation of anti-Candida T cells.** Because therapeutic strategies using anti-Candida T cells may require a high number of specific cells, we assessed whether the generated anti-Candida T cells could appropriately divide after stimulation

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with an endogenously processed antigen. When co incubated with autologous C. albicans–loaded APCs for 7 days, up to 55% of the isolated and expanded anti-Candida T cells proliferated as assessed by means of CFSE staining (n = 3) (figure 4). By contrast, <20% of anti-Candida T cells (mean, 11%) expanded during the same period when cocultured with unloaded APCs.

Alloreactive potential. Because immunotherapy with T cells may cause severe graft-versus-host disease (GVHD), we compared the alloreactive potential of purified anti-Candida T cells with that of CD4+ T cells of the original cell fraction. In all 3 individuals tested, up to 40% of the unselected CD4+ T cells elicited a strong proliferation signal against third-party APCs, whereas only a marginal expansion was observed in purified anti-Candida T cells coincubated with allogeneic APCs (figure 5). The loss of alloreactivity of selected anti-Candida T cells is also supported by significantly lower IFN-γ levels measured in the supernatant of anti-Candida T cells coincubated with third-party APCs (all levels were below the detection limit), compared with those of unselected CD4+ T cells coincubated with allogeneic APCs (median, 185 pg/mL [range, 98–325 pg/mL]; n = 3) (figure 5).

Antifungal activity of anti-Candida T cells. To assess the impact of generated anti-Candida T cells on hyphal damage of C. albicans, the colorimetric XTT assay was performed. In a total of 9 assays using 3 different purified T cell populations, expanded T cells alone exhibited marked hyphal damage (mean ± SE, 17.1% ± 14.7%) (figure 6). IFN-γ increased hyphal damage exhibited by PMNs only (10.5% ± 6.6% and 7.3% ± 5.6%, respectively). Stronger antifungal activity was seen when Candida hyphae were coincubated with expanded T cells and PMNs (23.1% ± 11.8%), but the combination of expanded T cells, APCs, and PMNs induced the highest antifungal damage (30.4% ± 15.3%) (figure 6).

DISCUSSION

Infections caused by Candida species are common fungal infections, in particular in patients undergoing alloHSCT [1]. Unfortunately, the mortality of invasive Candida infections has not improved over the past few years, despite the introduction of potent antifungal agents [3]. There is a growing body of evidence that T lymphocytes play a critical role in the host defense against fungi. Animal models have suggested that Th1 cells mediate phagocyte–dependent protection and represent the principal mediators of acquired protective immunity, whereas the production of inhibitory cytokines such as IL-4 and IL-10 by Th2 cells is associated with disease progression [6]. In this respect, dendritic cells that have been pulsed with Candida yeast are capable of inducing Th1-dependent antifungal resistance in nontransplanted mice and also provide protection in mice that have undergone allogeneic bone marrow transplant by accelerating the functional recovery of Candida–specific IFN-γ–producing CD4+ donor lymphocytes [8]. Because data have indicated that successful antifungal therapy may rely on the induction of an appropriate antifungal Th1 cell response [20], transferring Candida–specific immunity through the infusion of ex vivo–generated, donor-derived anti-Candida T cells might be a potent prophylactic or therapeutic approach in patients undergoing alloHSCT. The approach of adoptive immunotherapy has been well established for patients with CMV disease or EBV–associated posttransplantation lymphoproliferative disease, but there are few data on patients with IFIs [9–11]. This might be due, at least in part, to the complex antigenic properties of the fungal cell wall, which in Candida species consists of glucan, chitin, proteins, and glyco(manno)proteins [21]. Although several immunodominant antigens of Candida, such as a 65-kD mannoprotein or enolase, have been characterized [22, 23], for several reasons we used a cellular extract of C. albicans to generate anti-Candida T cells. First, the Candida cell wall is a dynamic structure with the ability to adapt to different environments and to differentially express antigens on its surface [24]. This antigenic variability may compromise the efficacy of T cells generated with a single cell–wall component or epitope. Second, we sought to increase the likelihood of generating functionally active T cells not only against C. albicans but also against other clinically relevant fungi. To this end, the administration of T cells that protect against an array of pathogens but that do not cause GVHD would be the ultimate goal in

Figure 6. Hyphal damage to C. albicans induced by anti-Candida T cells (ACTs), polymorphonuclear leukocytes (PMNs), and antigen-presenting cells (APCs), alone or in combination. Bars represent means ± SEs of 9 independent experiments. *P < .05 (analysis of variance followed by Dunnett’s correction for multiple comparisons). IFN, interferon.
the supportive care of transplant recipients. Interestingly, the T cells we generated with antigens derived from C. albicans also responded to stimulation with C. tropicalis but not to stimulation with C. glabrata, P. chrysogenum, Alternaria alternata, and Aspergillus fumigatus. Although unresponsiveness to molds was not surprising, the distinct pattern of activation with different Candida species was unexpected but might be explained by the fact that C. albicans has a closer phylogenetic relationship to C. tropicalis than to C. glabrata [25]. This is in line with the cross-reactivity of a number of antibodies of C. albicans with those of C. tropicalis but not with those of C. glabrata [26]. Spectratyping revealed that the T cells, which uniformly responded to antigens of both C. albicans and C. tropicalis, consist of various T cell subpopulations expressing different members of the Vβ family of the TCR. One can therefore conclude that multiple antigens of both fungi are capable of eliciting a uniform cellular immune response. However, these antigens are obviously different from antigens of C. glabrata and molds. Future studies have to address the detailed characterization of these antigens, which ultimately will provide new clues for the cellular immune response against Candida species.

In the present study, functionally active anti-Candida T cells produced IFN-γ but not IL-4 and IL-10 after restimulation, which indicated a Th1 response. It has been shown that dendritic cells discriminate between yeasts and hyphae of C. albicans and, thus, lead to a Th1 or a Th2 response, respectively [27]. Whereas a Th1 response is essential for the expression of protective immunity against Candida infection, a Th2 response exacerbates the severity of infection [7]. In contrast to CD4+ T cells, the importance of CD8+ T cells in invasive Candida infection is less clear [28, 29]. Although it has been suggested that CD8+ T cells play a minor role in Candida infection [30] and we did not observe intracellular production of IFN-γ by CD8+ lymphocytes, future experiments have to characterize the exact role of CD8+ T cells in the antifungal host response to Candida species as well a potential negative influence of immunosuppressive agents on anti-Candida T cells before adoptive immunotherapy can be applied in the clinical setting.

The number of anti-Candida T cells required for adoptive immunotherapy is unclear at present, but, according to results of studies of posttransplant viral disease, an adequate number of specific T cells might be crucial [31]. Because our data demonstrate that the generated anti-Candida T cells do not represent terminally differentiated CD4+ cells but that they proliferate after restimulation with Candida antigen, the adoptively transferred anti-Candida T cells may further expand if stimulated by Candida APCs in vivo. Importantly, cryopreservation did not affect any of the functional activities of the generated anti-Candida T cells. Therefore, it might be possible to generate these cells before transplant for patients at a very high risk of invasive candidiasis and to adoptively transfer these cells prophylactically or early during infection. The optimal time point for the adoptive immunotherapy, however, has to be determined in future studies.

It is important to note that the infusion of even small numbers of alloreactive donor-derived T cells may cause severe GVHD. In corroboration of reports about virus-specific T cells [32, 33], we observed that purified functionally active anti-Candida T cells marginally expanded when coincubated with allogeneic APCs, whereas a high percentage of unspecific and unselected CD4+ T cells elicited a strong proliferation response against third-party APCs. The marked reduction in alloreactivity is supported by significantly lower levels of IFN-γ in the supernatant of proliferating purified anti-Candida T cells than of proliferating unspecific T cells, given that IFN-γ is one of the hallmarks of acute GVHD [34].

Although the oxidative and nonoxidative pathways of PMNs against Candida species are well characterized [35], the effector mechanisms through which T cells participate in the control of fungal infection are not fully understood. In corroboration of previous reports on the antifungal effect of T cells [15, 36], we observed significant hyphal damage by T cells alone, which increased with increasing numbers of T cells (data not shown). Importantly, because of different periods of coin cubation in our setting, the antifungal activity of T cells cannot quantitatively be compared with the hyphal damage exhibited by PMNs. It has been shown that CD4+ CTLs are able to induce perforin-mediated cytotoxicity using lytic granules containing perforin and granzymes [37], but the mechanisms of Candida damage by T cells are the focus of our current research. We observed that the combination of PMNs, T cells, and APCs exhibited significantly more hyphal damage than each cell population alone or than the combinations of APCs with PMNs or T cells, respectively (P<.05). This finding might be explained by an increase in the antifungal activity of phagocytes by cytokines that were produced by anti-Candida T cells after the activation of APCs and by an additive antifungal effect of PMNs and T cells [38]. To this end, the direct and indirect mechanisms of hyphal damage by anti-Candida T cells have to be further evaluated, but our results suggest that the transfer of functionally active anti-Candida T cells could be beneficial for patients with impaired cellular immunity after alloHSCT.

In conclusion, we present a simple and feasible strategy for the generation of functionally active human T cells against Candida from a single blood draw. Before considering a clinical application, however, the efficacy and potential adverse effects of various numbers and schedules of anti-Candida T cells have to be assessed in animal models. In addition, the generation of anti-Candida T cells has to be modified in accordance with good manufacturing practices.
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References