Characterization of the Cellular Immune Responses to *Rhizopus oryzae* With Potential Impact on Immunotherapeutic Strategies in Hematopoietic Stem Cell Transplantation

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Infections due to mucormycetes have a poor outcome, in particular in allogeneic hematopoietic stem cell transplantation (HSCT). In order to evaluate the cellular host response against mucormycetes, we enriched and cultivated anti-*Rhizopus oryzae* T cells from healthy individuals. These cells were characterized as memory/effector Th1 cells, they proliferated upon restimulation, they exhibited cross-reactivity to some but not all Mucorales species tested, and they increased the activity of phagocytes. Compared with the original cell fraction, the generated cells exhibited significant lower alloreactivity. Our data may form the basis for further investigations, which may ultimately lead to adoptive immunotherapeutic strategies for allogeneic HSCT recipients suffering from mucormycosis.

Mucormycosis increasingly occurs in hematopoietic stem cell transplant (HSCT) recipients [1, 2]. A prospective surveillance study reported that mucormycosis, usually caused by the genera *Rhizopus, Rhizomucor, Mucor*, and *Lichtheimia*, was diagnosed in almost 8% of HSCT recipients suffering from invasive fungal disease (IFD), with a 1-year mortality of 72% [3]. Although neutropenia is one of the major risk factors for mucormycosis [2], the roles of other host defense mechanisms, such as the T-cell response, are not well characterized. In contrast, it is well known that Th1-biased immunity correlates with protective immunity against *Aspergillus* and *Candida* [4], which is the rationale for adoptively transferring antifungal T cells to allogeneic HSCT recipients to enhance pathogen-specific immunity and thereby limit infectious complications. We hypothesized that T cells might also play a role in immunity against *Rhizopus oryzae*, and we thus aimed to isolate and characterize antigen-specific T cells against this pathogen in order to develop immunotherapeutic strategies in the setting of allogeneic HSCT.

**MATERIALS AND METHODS**

**Study Subjects**

After informed consent, 100 mL of peripheral blood was obtained from healthy volunteers for the isolation of anti-*R. oryzae* T-cells and the preparation of monocyte-derived autologous antigen-presenting cells (APCs). None of the individuals had a history of IFD or atopic disease. The protocol was approved by the ethics committee of Frankfurt.

**Fungal Antigens**

The water-soluble cell extract of *R. oryzae* (clinical isolate, University of Innsbruck) was prepared as previously described [5]. For testing specificity, cellular extracts of 2 strains of *Rhizopus microsporus* and 1 strain each of *Rhizomucor pusillus, Mucor circinelloides* (all clinical isolates, University of Innsbruck), *Candida albicans* (CA444), *Aspergillus fumigatus* (CBS144.89), *Aspergillus flavus* (CBS56965) and *Alternaria alternata* (IP1563) were used.

**Isolation and Expansion of Anti–*R. oryzae* T Cells**

A total of 1 × 10^8 peripheral blood mononuclear cells (PBMCs) were stimulated for 16 hours at a concentration of 1 × 10^7/mL with 7.5 µg/mL *R. oryzae* cell extract in cytotoxic T lymphocyte (CTL) medium containing Roswell Park Memorial Institute 1640 medium (Gibco), 1 µg/mL ciprofloxacin (Fresenius Kabi), 1 ng/mL tobramycin (Infectopharm), and 10% pooled heat-inactivated human serum. Then, activated T cells were isolated using the CD154 MicroBead Kit according to the manufacturer’s instructions (Miltenyi Biotec) and placed in a 25-cm^2_ cell culture flask (Corning) in 10-mL CTL medium containing 50 IU/mL of recombinant human...
interleukin 2 (rhIL-2) (Chiron) and irradiated, autologous R. oryzae-loaded APCs. Cultures were supplemented with fresh medium and 50 IU/mL of rhIL-2 every other day for up to 14 days and stimulated with irradiated autologous R. oryzae-loaded APCs on days 4, 7, and 10. In a second isolation step, generated T cells were stimulated with APCs previously stimulated with antigens of various fungi, including mucormycetes, as previously described [6]. For testing cross-reactivity, generated T cells were stimulated with APCs previously stimulated with antigens of various fungi, including mucormycetes, Aspergillus spp., and yeasts (7.5 µg/mL each).

Characterization of Phenotype and Intracellular Cytokine Staining

Phenotyping of purified T cells was performed by an 8-color flow-cytometer (FACS CantoII; BD Biosciences) using monoclonal antibodies against CD3, CD4, CD8, CD14, CD19, CD45, CD45RO, CD56, CD62L, CD154, HLA-DR, IFN-γ, and tumor necrosis factor α (TNF-α), as indicated (BD Biosciences). Intracellular cytokine flow cytometry was performed as previously described [6]. For testing cross-reactivity, generated T cells were stimulated with APCs previously stimulated with antigens of various fungi, including mucormycetes, Aspergillus spp., and yeasts (7.5 µg/mL each).

Carboxyfluorescein succinimidyl ester (CFSE) Staining

CFSE (Molecular Probes) staining was performed as previously described [6]. For testing proliferation on restimulation, irradiated autologous APCs, either unstimulated or antigen-stimulated, were added. For testing alloreactivity, irradiated allogeneic APCs were added to labeled PBMCs or to labeled anti-R. oryzae T cells. On days 0 and 7, cells were stained with CD3 and CD4 and analyzed by means of flow cytometry.

Oxidative Burst

Oxidative burst was assessed using the Bursttest (Phagoburst; Orpegen Pharma) according to the manufacturer's instructions with some modifications. In brief, 100 µL of supernatant of anti-R. oryzae T cells coincubated either with Rhizopus-loaded APCs or unstimulated APCs or alone were added to 100 µL of fresh blood. As negative and positive controls, medium alone or medium supplemented with phorbol 12-myristate 13-acetate (PMA) was added to the blood, respectively. The turnover of the substrate indicating the phagocytic activity was analyzed by means of flow cytometry.

Cytometric Bead Array

Concentrations of IFN-γ, TNF-α, interleukin 4 (IL-4), and interleukin 17 (IL-17) in the supernatant were determined using the BD Cytometric Bead Array (CBA) Flex Set System according to the manufacturer's instructions (BD Biosciences). The lower limits of detection were 1.8 pg/mL (IFN-γ), 0.7 pg/mL (TNF-α), 1.4 pg/mL (IL-4), and 0.3 pg/mL (IL-17).

Cross-Reactivity of Anti-R. oryzae T Cells

Coincubation of anti-R. oryzae T cells with APCs loaded with fungal antigens resulted in IFN-γ response upon stimulation with R. microsporus (both var. microsporus and var. oligosporus), R. pusillus, and M. circinelloides. In addition, the generated T cells responded to APCs loaded with antigens of A. fumigatus, Penicillium chrysogenum, and C. albicans, whereas

RESULTS

Isolation and Cultivation of Human T Cells Responding to R. oryzae Antigens

To assess potential cellular immune responses to R. oryzae, we stimulated PBMCs with antigens of R. oryzae and observed in all 6 individuals tested IFN-γ production in CD4+ T cells (0.1%–1.2%) (data not shown). This small number of anti-R. oryzae T cells was enriched and cultivated for further characterization. Flow cytometry revealed a highly homogenous population of CD4+ T cells (mean, 94.4%; range, 90.1%–96.7%; n = 6), whereas only marginal numbers of CD8+ (mean, 2.5%), CD19+ (mean, 0%), CD3+CD56+ (mean, 0.6%), and CD3+CD56- cells (mean, 1%) were detected. The cells homogeneously expressed HLA-DR and CD45RO (>97%), indicating an activated memory T-helper cell population. Because upon restimulation, nearly all of the cells (>99%) lost CD62L, these cells could be further specified as activated effector/memory T cells.

Generated Anti-R. oryzae T Cells Respond Upon Restimulation With Cytokine Production and Proliferation

When coincubating anti-R. oryzae T cells with autologous R. oryzae-loaded APCs, a significant percentage of cells produced IFN-γ and TNF-α upon restimulation, as demonstrated by flow cytometry (median IFN-γ, 6.3%; range, 1.5%–26.6%; median TNF-α, 14%; range, 3.4%–34.5%; n = 6). In contrast, no significant IL-4 and interleukin 10 production was seen (data not shown). Similarly, a strong increase of IFN-γ concentration was measured in the supernatant (median, 269 pg/mL; range, 37–423), indicating an activated memory T-helper cell population. Because upon restimulation, nearly all of the cells (>99%) lost CD62L, these cells could be further specified as activated effector/memory T cells.

Statistical Analysis

Data were analyzed using GraphPad Prism (version 4.00; GraphPad Software) and compared by Mann–Whitney or unpaired t test, where applicable. Two-sided P < .05 was considered to be statistically significant.
no response was seen upon APCs loaded with antigens of A. flavus, A. alternata, and Mucor racemosus (Figure 1).

**Decreased Alloreactivity of Anti–R. oryzae T Cells**

Alloreactivity was evaluated by proliferation and IFN-γ secretion. In all 3 experiments, coincubation of unselected CD4⁺ cells with allogeneic APCs elicited a strong proliferation response compared with that without allogeneic APCs (median, 32.4%; range, 13.3%–54.2%; vs median, 3.7%; range, 3.3%–4.1%). In contrast, the expansion of anti–R. oryzae T cells was only marginally increased by coincubation with allogeneic APCs (median, 27.3%; range, 24.7%–29.0% with allogeneic APS; vs median, 29.3%; range, 22.6%–35.3% without allogeneic APCs) (Supplementary Figure 2). The loss of alloreactivity was also supported by significantly higher IFN-γ levels in the supernatant of unselected CD4⁺ cells coincubated with allogeneic APCs than those measured in the supernatant of anti–R. oryzae T-cells incubated alone or coincubated with allogeneic APCs and of unselected CD4⁺ cells incubated alone (median levels, 184, 19, 34, and 12 pg/mL, respectively).

**Stimulated Anti–R. oryzae T Cells Enhance Phagocyte Activity**

The effect of anti–R. oryzae T cells on phagocytes was assessed by assessing oxidative burst activity. When adding the supernatant of anti–R. oryzae T cells coincubated with R. oryzae–loaded APCs to granulocytes, the activity of granulocytes was significantly higher (mean, 5.32%; standard deviation [SD], 1.68%) than when the supernatant of anti–R. oryzae T cells...
incubated alone (mean, 0.16%; SD, 0.38%) or the supernatant of anti–R. oryzae T-cells coincubated with unstimulated APCs (mean, 0.8%; SD, 0.25%) (<i>P</i> < .001) was added (Figure 2). Similar results were observed when investigating the oxidative burst activity of monocytes (mean, 3.9%; SD, 0.57%; vs mean, 0.35%; SD, 0.07%; and mean, 1%; SD, 0.14%, respectively; <i>P</i> < .02 each, respectively; data not shown).

**DISCUSSION**

Despite major improvements in supportive care, mucormycosis still has a poor outcome, in particular in allogeneic HSCT recipients [3]. Unfortunately, in contrast to IFDs due to Candida and Aspergillus spp in which T-cell immunity has been extensively studied [6–8], little is known about cellular immunity against mucormycetes. However, the observation that mucormycosis occurs in a significant number of allogeneic HSCT recipients at a time when neutropenia and mucositis have resolved but adaptive immune responses are still hampered [9, 10] suggests a potential role of T cells in the host response to mucormycetes.

Our preliminary experiments revealed T-cell responses upon <i>R. oryzae</i> in the peripheral blood of all healthy individuals tested, which is not surprising because <i>R. oryzae</i> is ubiquitous. In these individuals, the frequency of anti–<i>R. oryzae</i> T cells was low and in the range of that observed in T cells responding to Candida and Aspergillus (TL, unpublished observations) [11]. However, we could generate with our strategy a large number of anti–<i>R. oryzae</i> T cells, which predominantly consisted of activated memory T<sub>H1</sub> cells. Numerous studies have identified the pivotal role of T<sub>H1</sub> immunity in the clearance of various IFDs [4], and in the clinical setting, restoring antifungal T<sub>H1</sub> immunity by the adoptive transfer of anti-Aspergillus T<sub>H1</sub> cells has already shown therapeutic efficacy in allogeneic HSCT recipients [12]. Interestingly, the generated cells consisted of a small but significant number of cells producing IL-17, which mobilizes neutrophils, induces the production of defensins, and contributes to the efficient control of an infection [4]. Corroborating previous results in Aspergillus and Candida [6, 7], we did not detect a significant number of CD8<sup>+</sup> T cells among the generated anti–<i>R. oryzae</i> T cells.

The generated functionally active anti–<i>R. oryzae</i> T cells do not represent terminally differentiated CD4<sup>+</sup> cells, but they proliferate upon restimulation with antigens of <i>R. oryzae</i>, which might have important clinical implications. Because it seems likely that the generated T cells further expand in vivo if stimulated by <i>R. oryzae</i>–loaded APCs, even small numbers of transferred T cells may be sufficient for antifungal immunotherapy. However, the exact number of T cells required has to be determined.

Interestingly, when generating antifungal T cells with antigens of <i>R. oryzae</i>, these cells showed cross-protection against a variety of filamentous fungi and yeasts. However, we did not observe overall antimucormycete reactivity because the generated cells did not respond to <i>M. racemosus</i>. This indicates that a number of fungi across species and genera share common epitopes, which is supported by a recent report that the cell wall glucanase Crf1 of <i>A. fumigatus</i> induces memory CD4<sup>+</sup> T<sub>H1</sub> cells that are cross-reactive to <i>C. albicans</i> [8]. On the other hand, the epitopes to our antigen extract are not shared by all Mucorales. Further studies have to further evaluate potential cross-reactivity because adoptive administration of a T-cell population that protects against an array of medical important fungi would be the ultimate goal in the supportive care of transplant patients.

Because immunotherapy with T cells can cause severe graft-versus-host disease (GVHD), it is important to note that assessment of both proliferation and IFN-γ production as a hallmark of GVHD indicates that the purified anti–<i>R. oryzae</i> T cells demonstrate significantly less alloreactivity than the original cell fraction. These data corroborate previous reports about in vitro–generated virus-specific and antifungal T cells [6, 7, 13].

The significant increase of phagocyte oxidative burst activity by the generated cells might be due, at least in part, to the elevated levels of IFN-γ, which is known to augment...
antifungal host response mechanisms [14]. In this study, we did not address potential direct damage of \textit{R. oryzae} by the generated CD4$^+$ cells, which has been described for \textit{Aspergillus} and \textit{Candida} [6, 7].

In conclusion, we demonstrate that a low but significant number of anti–\textit{R. oryzae} T cells can be detected in healthy individuals. After isolation and cultivation, the activated anti–\textit{R. oryzae} T$_{41}$ cells proliferate upon restimulation, demonstrate cross-reactivity to some other fungi, increase the antifungal activity of phagocytes, and exhibit significantly lower alloreactivity than the original cell fraction. Our data may form the basis for further investigations, which may ultimately lead to adoptive immunotherapeutic strategies for allogeneic HSCT recipients suffering from mucormycosis.

Supplementary Data

Supplementary materials are available at the Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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