A New Method for Reactivating and Expanding T Cells Specific for Rhizopus oryzae

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Mucormycosis is responsible for an increasing proportion of deaths after allogeneic bone marrow transplantation. Because this disease is associated with severe immunodeficiency and has shown resistance to even the newest antifungal agents, we determined the feasibility of reactivating and expanding Rhizopus oryzae-specific T cells for use as adoptive immunotherapy in transplant recipients. R. oryzae extract-pulsed monocytes were used to stimulate peripheral blood mononuclear cells from healthy donors, in the presence of different cytokine combinations. The generated R. oryzae-specific T cell products were phenotyped after the third stimulation and further characterized by the use of antibodies that block class I/II molecules, as well as pattern recognition receptors. Despite the very low frequency of R. oryzae-specific T cells of healthy donors, we found that stimulation with interleukin-2 (IL-2)/IL-7 cytokine combination could expand these rare cells.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is the only curative option for many hematologic malignancies that are refractory to standard chemotherapies and radiotherapies.1 However, opportunistic infections are a major cause of morbidity and mortality post-transplant,2 owing to the immune suppression required to facilitate engraftment and prevent graft-versus-host disease (GVHD).3

Mucormycosis is a devastating invasive fungal disease caused mainly by Rhizopus oryzae.4 It affects both children and adults with poorly controlled diabetes, iron overload, major trauma, or severe immunosuppression, as seen after HSCT,1 and accounts for 8%–13% of all invasive fungal infections.4,6 This frequency is increasing7 as a result of improved diagnostic techniques and resistance to agents given as first-line treatment to prevent invasive fungal infections in immunocompromised hosts.8–11 Even with aggressive surgical interventions and the use of drugs, recommended based on their in vitro activity, such as amphotericin B, posaconazole, and isavuconazole,12,13 the mortality rate caused by mucormycosis in patients undergoing HSCT has been reported to be at least 75%, which is considerably higher than that reported for other invasive fungal diseases.14,15 Hence there is an urgent need for novel approaches to the prevention and treatment of this disease after HSCT.

Studies of other opportunistic infections occurring posttransplant, particularly viral infections, have demonstrated the crucial role of adaptive immune reconstitution in disease recovery, leading to the use of adoptive T cell therapies to prevent or treat infections associated with adenovirus, Epstein-Barr virus (EBV), cytomegalovirus (CMV), BK virus, and human herpesvirus 6 (HHV6).16–18 Whether T cells have a role in protective immunity against mucormycosis remains unclear. Although neutrophils play essential roles in the elimination of this pathogen, there is some evidence that the innate immune response is less effective against Mucorales species than other filamentous fungi, because mucor fungal infections have been reported in HSCT recipients, even after normal neutrophil numbers have recovered.19,20 This strongly suggests that other immune components contribute to the defense against mucormycosis,21 and some groups have suggested that Mucorales-specific T cells with in vitro activity against hyphae persist in transplant patients with invasive mucormycosis until the infection has resolved.22–24 These
antigens are at extremely low levels, we harvested supernatants from viable fungal cultures. To con

R. oryzae (mean 160 IFN-γ/cells; Figure 1), even when the PBMCs were exposed to mucor antigen, range 0–24.5 (Figure S1). These findings suggest that memory R. oryzae responses, like other fungal-T cell responses, are present at very low frequencies, if at all, in agreement with observations by other groups.

R. oryzae-Specific T Cells from Healthy Donors Can Be Expanded In Vitro

To determine the feasibility of expanding T cells specific for R. oryzae in vitro, we modified a protocol that had been successfully used to expand rare antigen-specific T cells from healthy donors (e.g., tumor-specific T cells) in sufficient numbers to be useful as adoptive immunotherapy.10,21 We first evaluated different cytokine combinations (interleukin-2 [IL-2]/IL-7, IL-4/IL-7, IL-7/IL-15, and IL-15/IL-21) from three healthy donors to identify an effective combination in terms of specificity and T cell expansion potential. The three combinations containing IL-7 produced comparable results (mean 5-fold expansion after two stimulations, range 4.5–6.5), whereas T cells grown in the IL-15/IL-21 combination failed to expand (Figure 2A). The majority of T cells expanded in the IL-7-containing combinations were CD4+ (mean frequency 64.1%, 75.0%, and 48.8%, respectively) with a memory phenotype (mean frequency of CD45RA+ CD45RA− T cells is 62.6%; Figure 2B). All cytokine combinations elicited T cells specific for R. oryzae as measured by IFN-γ enzyme-linked immunospot (ELISpot) assay (Figure 2C). After the third stimulation, the mean response of T cells expanded in IL-2/IL-7 was 1.127 IFN-γ SFCs/1 × 10⁵ cells, in IL-4/IL-7 was 107 IFN-γ SFCs/1 × 10⁵ cells, in IL-7/IL-15 was 988 IFN-γ SFCs/1 × 10⁵ cells, and for IL-15/IL-21 it was only 28 IFN-γ SFCs/1 × 10⁵ cells, with background responses to unpulsed monocytes of 28, 7, 112, and 2.4 IFN-γ SFCs/1 × 10⁵ cells for each cytokine combination, respectively. T cells expanded in IL-2/IL-7 and IL-7/IL-15 showed greater R. oryzae specificity than did T cells grown in the presence of IL-4/IL-7 or IL-15/IL-21 (Figure 2C). Thus, on balance, stimulation with the IL-2/IL-7 combination appears to offer the most effective strategy for generating R. oryzae-specific T cells; therefore, we used this combination for all further experiments.

Expanded Bulk T Cell Products, Containing R. oryzae-Specific T Cells, Are Predominantly CD4+ T Cells with Memory Phenotype

We next sought to determine the reproducibility of this method before its validation in the good manufacturing practice (GMP) setting. After expanding R. oryzae-specific T cells from eight healthy donors in IL-2/IL-7-containing medium, we observed a mean 31.2-fold expansion on day 23 after the initial stimulation (range 7- to 52-fold expansion; n = 8; Figure 3A). The mean percentage of CD4+ T cells of the expanded bulk T cell product, containing R. oryzae-specific T cells, was 50% (range 17%–83%; n = 8; Figure 3B), with the majority of T cells showing memory phenotype (mean 69.25% CD28, 77.9% CD45RA). All expanded T cell products showed specificity for R. oryzae antigens by ELISpot with a mean of 344 IFN-γ SFCs/1 × 10⁵ cells (range 57–1,057; n = 10) compared with a mean observations indicate that adoptive T cell therapy might be useful in the management of mucormycosis post-HSCT. Indeed, T cells reactive to fungi in the order Mucorales have been identified in infected patients and isolated from the peripheral blood of healthy donors, enabling investigators to generate fungus-reactive T cells for potential clinical use.15,26 Despite the availability of these methods for activation and expansion of T cells specific for R. oryzae, more comprehensive characterization of the process and the products is needed. Thus, we sought to isolate, expand, and characterize T cells specific for R. oryzae, the most common fungus found in patients with mucormycosis.6,8,27,28

Figure 1. Frequency of Fungi-Reactive T Cells in Healthy Donor PBMCs

Using peripheral blood from healthy donors (n = 9), we evaluated the lymphocyte responses to R. oryzae (Mucor lys), A. fumigatus (Asp lys), CMV, and EBV using IFN-γ ELISpot assays. Each symbol represents the mean number of spot-forming cells from at least two replicates containing 500,000 cells/well. Bars denote the means. *p < 0.05; **p < 0.01.
Figure 2. Optimization of Expansion Conditions
(A) Comparison of the expansion of T cell products derived from three healthy donors. T cells were stimulated with the cytokine combinations IL-2/IL-7, IL-4/IL-7, IL-7/IL-15, or IL-15/IL-21. Each dot represents the mean fold expansion, and error bars denote SD. (B) Phenotype of three healthy donors expanded with the cytokine combinations IL-2/IL-7 versus IL-4/IL-7 versus IL-7/IL-15, versus IL-15/IL-21. All subsets were gated on CD3+ cells, with the exception of non-T cell subsets (CD3-CD56+ and CD3-CD19+), which were gated on singlets from total lymphocytes. Bars represent means and SDs. (C) Antigen specificities of T cell products derived from three healthy donors as measured by IFN-γ secretion. T cells were stimulated three times with the cytokines IL-2/IL-7 versus IL-4/IL-7 versus IL-7/IL-15, versus IL-15/IL-21. Negative control denotes unpulsed APCs (monocytes alone), whereas Mucor denotes pulsed APCs (monocytes plus R. oryzae lysate). Bars indicate mean values.

Figure 3. Characterization of R. oryzae-Specific T Cells Expanded with IL-2/IL-7
(A) Mean fold expansion and SDs of antigen-specific T cells derived from healthy donors after 3 stimulations with APCs pulsed with R. oryzae lysate (n = 8). (B) Phenotype of the T cell products. All subsets are gated on CD3+ cells (n = 8). Bars indicate means and SDs. (C) Specificity of T cell products against R. oryzae (p < 0.01) in an ELISPOT assay comparing T cell responses to unpulsed (monocytes alone) versus pulsed (monocytes + R. oryzae lysate) targets. Each symbol denotes a healthy donor (n = 8). **p < 0.01.
of 18 IFN-γ SFCs/1 × 10⁵ cells (range 1–95; n = 10) in response to unpulsed monocytes (p = 0.0069; Figure 3C). Specificity was also determined by release of perforin and granzyme B in response to R. oryzae antigens (Figure S2A), as well as simultaneous measurements of IFN-γ, IL-2, granzyme B, and tumor necrosis factor alpha (TNF-α) using a FluoroSpot assay (Figures S2B and S2C).

**R. oryzae-Specific T Cells Recognize Fungal Antigens through Class II Presentation**

The fungal antigens used in this study were derived from whole lysate from R. oryzae, which introduces potential activation signals that rely on pattern recognition receptors (PRRs),21 as well as from fungal antigens loaded on MHC molecules. To distinguish between these two possibilities, we performed ELISpot assays in which we added antigens (Figure S2A), as well as simultaneous measurement of IFN-γ, IL-2, granzyme B, and tumor necrosis factor alpha (TNF-α) using a FluoroSpot assay (Figures S2B and S2C).

**R. oryzae-Specific T Cells Secrete Multiple Cytokines**

R. oryzae-specific T cells secreted multiple cytokines in addition to IFN-γ after three stimulations. T cells were stimulated with monocytes presenting R. oryzae antigens in the presence of IL-2 and IL-7, and supernatants were harvested after 24 hr. In three evaluated lines, the T cells secreted IL-5 (mean 3,331 pg/mL, range 112–9,636), IL-10 (mean 383 pg/mL, range 9–831), IL-13 (mean 9,522 pg/mL, range 999–19,934), and TNF-α (mean 776 pg/mL, range 196–1,156) (n = 3) (Figure 5). By contrast, secreted levels of IL-17A, IL-6, and IL-21 were negligible; measurements of other cytokines are shown in Table 1. Furthermore, we showed that cytokines were detected only in the presence of antigens (Figure S3A) and cytokine secretion of IFN-γ/IL-13/IL-5, except TNF-α (which may be secreted by NK cells present at the beginning of culture) was markedly increased after three stimulations compared with the beginning of the culture (Figure S3B).

**DISCUSSION**

In this report, we demonstrate the feasibility of expanding low-frequency R. oryzae-specific T cells to populations adequate for clinical testing of safety and efficacy. The cytokine combination of IL-2 and IL-7 yielded a greater total expansion of antifungal T cells than did IL-7/IL-15 (Th1), IL-4/IL-7 (Th2), or IL-15/IL-21. The expanded T cell products consisted mainly of CD4+ T cells with a predominant memory phenotype and showed HLA-restricted specificity for R. oryzae by IFN-γ ELISpot assay and HLA blocking assay. Finally, the R. oryzae-specific T cells secreted cytokines other than IFN-γ, associated with antifungal immunity.21,23

Our stimulation strategy, which differs from those of other groups,32 centers on the use of monocytes rather than dendritic cell (DC) as antigen-presenting cell (APC) and on frequent cytokine support with IL-2 and IL-7. By taking advantage of monocytes and all the co-stimulatory molecules physiologically needed by PBMCs, we overcome the tedious and challenging process of producing DCs in vitro. This approach generates R. oryzae-specific T cells that secrete Thelper 2 (Th2) cytokines in addition to Th1 and regulatory T cell (Treg) cytokines (but not Th17; see Table 1). To determine the optimal cytokine combination to expand fungus-specific T cells, we compared various cytokine regimens to identify the cytokine cocktail inducing the highest yield of specific T cells. Although the cytokine support (i.e., IL-2 and IL-7) we describe may be optimal for the ex vivo expansion of rare fungus-specific T cells, it may not be relevant to a larger starting
population of T cells specific for other pathogens (e.g., in the CMV setting). The cytokine cocktail that proved optimal for eliciting a T cell response to *Rhizopus* antigens differed from the cocktails required to expand: (1) virus-specific T cells from healthy seropositive donors (IL-4/IL-7), 17 (2) alloreactive T cells (IL-7/IL-15), 24 and (3) tumor antigen-specific T cells (IL-7/IL-12/IL-15 or IL-15/IL-21). 31,35 These discrepancies underscore the need for more comprehensive analyses that could be used to generate antifungal T cells. From a manufacturing perspective, this would help to clarify the feasibility of generating multipathogen-specific T cells and may identify combinations of pathogen antigens that can be used in the same culture, as well as those that cannot. Although our manufacturing strategy yields a relatively modest fold expansion (up to 50-fold), this is consistent with what other groups have observed. 36 Moreover, similar expansions can be observed irrespective of whether peptide mixtures or antigen extracts were used. 36 Interestingly, we also observed that *R. oryzae*-specific T cell products were cross-reactive with other fungal pathogens. Specifically, responses to *Aspergillus fumigatus* antigens were detected as shown in Figure S4.

We anticipate that the generation of *R. oryzae*-specific T cells may require higher initial numbers (e.g., from leukapheresis collections) to obtain clinically relevant cell numbers for infusion. However, we also predict that the starting dose will be similar to that used in the virus-specific T cell setting (5 × 10^6-/m^2 to 2 × 10^7-/m^2). 18 We posit that patients who have recovered from mucormycosis after bone marrow transplantation (BMT) have higher initial percentages of memory T cells against *R. oryzae*. Hence to accelerate memory immune reconstitution to *R. oryzae*, we propose to administer donor-derived *R. oryzae*-specific T cells to high-risk patients early (during the neutropenic phase) after BMT to prevent and treat fungal disease.

It has been suggested that there are many potential roles for T cells in combating fungi, from stimulation of the innate immune response 21 to the potential direct induction of hyphal damage. 22 Unfortunately, the vast majority of these proposals apply to *Candida* or *Aspergillus* species, with only a minority directed to *Rhizopus* species. Although we do not provide direct evidence of antifungal activity by the activated T cells following our manufacturing methodology, the profile of secreted cytokines suggests that the expanded T cells would recognize fungi. Indeed, we show that *R. oryzae*-specific T cells secrete a broad range of cytokines that have all been implicated in the immune response to fungi (Figure 5; Table 1). 23 IFN-γ has long been associated with protection against fungi, 21 whereas a polymorphism that imparts low production of IL-10 has been associated with a poor response to invasive aspergillosis. 38 Further, anti-TNF-α therapies are thought to contribute to the risk for disseminated mucormycosis, 23 whereas the early clearance of *R. oryzae* antigen relies on activation of eosinophils by IL-5. 25 Moreover, the activation of liver receptor homolog-1 (LRH-1) by IL-13 in macrophages confers an antifungal phenotype to these cells, because animals deficient in LRH-1 are highly susceptible to fungal infection. 41 Finally, it is biologically critical to demonstrate whether the T cell responses are induced in a nonspecific versus specific manner that has not been clearly evaluated in the past. In this study, we found specific antifungal immunity by demonstrating activity mainly through MHC class II using HLA/PRR blocking assays. To the best of our knowledge, this is the first study to show that after multiple stimulations, *R. oryzae*-specific T cell responses are progressively selected in the cultures. However, whether T cells important in controlling *R. oryzae* infections must be antigen specific will only be established in human clinical trials using *R. oryzae*-specific T cells.

By demonstrating the feasibility of T cell production specific for *R. oryzae*, using a robust and reproducible method with very small quantities of starting memory T cells, we have opened a new route for the development of adoptive T cell therapy directed to *R. oryzae*-induced mucormycosis and perhaps to other invasive fungal diseases as well.

**MATERIALS AND METHODS**

**Healthy Donor Samples**

PBMCs were obtained from discarded buffy coat samples from the blood bank at the NIH or from healthy donors at Baylor College of Medicine or Children’s National Health System, who gave their informed consent in accordance with the policies of their local institutional review boards.

**Screening for Fungal Antigen-Specific Responses**

PBMCs were isolated from peripheral blood using Ficoll density gradient centrifugation, as previously described. 17 They were then plated in duplicate wells in an IFN-γ ELISpot plate at 500,000 cells/well and stimulated with medium alone, *R. oryzae* lysate (*R. oryzae* grown in enriched trypticase; Greer Laboratories, Lenoir, NC, USA), *Aspergillus* extract (A. fumigatus; Greer Laboratories), EBV peptides (a combination of EBNA1, EBNA3a, EBNA3b, EBNA3c, EBNA5a, macrophage inflammatory protein-3).

| Table 1. Cytokine Expression of *Rhizopus* oryzae-Specific T Cells |
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| **Donor** | **IL-17F** | **GM-CSF** | **MIP3α** | **IL-12p70** | **IL-22** | **IL-9** | **IL-1b** | **IL-33** | **IL-4** | **IL-23** | **IL-6** | **IL-17E/IL-25** | **IL-27** | **IL-31** | **TNF-β** | **IL-28A** |
| **1** | ND | 5.70 | 76.13 | 10.32 | 0.05 | 19.41 | 14.45 | ND | 2.51 | ND | 20.99 | ND | ND | ND | 0.05 | ND |
| **2** | 1.23 | 10.67 | 35.51 | 6.72 | 0.14 | 10.93 | 6.08 | ND | 0.03 | ND | 23.50 | ND | ND | ND | ND | ND |
| **3** | ND | 1.73 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |

Values are presented as pg/mL. ND, not detected/below the limit of detection; GM-CSF, granulocyte-macrophage colony-stimulating factor; CCL20, chemokine (C-C motif) ligand 20; MIP3α, macrophage inflammatory protein-3.
subsequent stimulations. A combination of IL-2 and IL-7 for the optimized process, T cell cultures were stimulated with a cytokine assay. Each sample was plated in duplicate wells. Monocytes for antigen processing and presentation were isolated by CD14 magnetic MACS selection (Miltenyi, Bergisch Gladbach, Germany) and antigenic MACS selection (Miltenyi, Bergisch Gladbach, Germany) and 5% human AB serum (Gemiini Bioproducts, West Sacramento, CA, USA), and 2 mmol/L GlutaMAX (GIBCO, Gaithersburg, MD, USA) for 10–11 days. R. oryzae and A. fumigatus lysate were manufactured by Greer laboratories, using methods similar to their production of other fungal extracts: fungi are statically grown, extracted with ammonium bicarbonate, diazylated against distilled water, defatted, powdered, and dried (Greer Laboratories). For optimization experiments, T cell cultures were initially maintained in medium containing different cytokine combinations: IL-2/IL-7, IL-4/IL-7, IL-7/IL-15, or IL-15/IL-21 (Greer Laboratories). For development, the plates were washed in PBS/0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) or mucor lysate with monocytes. FluoroSpot, with the appropriate positive controls: SEB (Sigma-Aldrich) and -CD16 (BD Biosciences, San Jose, CA, USA). Blocking of MHC class I and II was performed as described above. The human 4-color FluoroSpot Kit was used to measure for secretion of IFN-γ, IL-2, TNF-α, and granzyme B by Mucor-specific T cells (Immunospot, Cleveland, OH, USA). Cells were plated at 1e5 cells/well on a 4-color FluoroSpot, with the appropriate positive controls: SEB (Sigma Aldrich, St. Louis, MO, USA) or mucor lysate with monocytes. FluoroSpot plates were developed according to the kit protocol and sent for unbiased spot counting to Immunospot.

Generation of R. oryzae-Specific T Cell Lines
After overnight culture to activate monocytes (monocytes are adhered on plastic for at least 12 hr, as previously described),12 PBMCs were pulsed with 0.8–4 μg R. oryzae lysate per 1 × 10^6 PBMCs for 1–2 hr and then cultured in Hyclone RPMI 1640 (Fisher Scientific, Hampton, NC, USA) supplemented with 45% Clicks media (Irvine Scientific, Santa Ana, CA, USA), 5% human AB serum (Gemiini Bioproducts, West Sacramento, CA, USA), and 2 mmol/L GlutaMAX (GIBCO, Gaithersburg, MD, USA) for 10–11 days. R. oryzae and A. fumigatus lysate were manufactured by Greer laboratories, using methods similar to their production of other fungal extracts: fungi are statically grown, extracted with ammonium bicarbonate, diazylated against distilled water, defatted, powdered, and dried (Greer Laboratories). For optimization experiments, T cell cultures were initially maintained in medium containing different cytokine combinations: IL-2/IL-7, IL-4/IL-7, IL-7/IL-15, or IL-15/IL-21 (R&D, Minneapolis, MN, USA). After 10–11 days, responder T cells were restimulated with R. oryzae-pulsed plastic adherence-activated monocytes (PBMCs were plated overnight to allow plastic adherence of monocytes) pulsed with 0.8–4 μg R. oryzae lysate per 1 × 10^6 PBMCs) for 1–2 hr at a ratio of 1:1 in the presence of cytokine combinations, as indicated, and fed with media containing 50 IU/mL IL-2 on days 3 and 5 following the second stimulation. R. oryzae-pulsed monocytes were irradiated prior to co-culture with T cells to avoid nonspecific T cell growth. Every week thereafter, responder T cells were stimulated in a similar fashion for a total of three stimulations. For all other expansions following identification of the optimized process, T cell cultures were stimulated with a cytokine combination of IL-2 and IL-7 for the first stimulation and during subsequent stimulations.

T Cell Phenotyping
The phenotype of the expanded bulk T cell products after the third stimulation was determined by surface staining with anti-CD4, -CD8, -CD3, -CD45RA, -CD45RO, -CCR7, -CD25, -CD56, and -CD16 (BD Biosciences, San Jose, CA, USA). All cells were analyzed on a BD Accuri, a BD FACSCalibur, or a Miltenyi MACSQuant flow cytometer. Control samples labeled with appropriate isotype-matched antibodies or unstained controls were included for each experiment and were used to define flow gates and quadrants.

IFN-γ ELISpot Assay
R. oryzae extract recognition was tested in an IFN-γ ELISpot assay. Each sample was plated in duplicate wells. Monocytes for antigen processing and presentation were isolated by CD14 magnetic MACS selection (Miltenyi, Bergisch Gladbach, Germany) and incubated in DC medium for 18 hr to allow activation via plastic adherence. Monocytes were pulsed with A. fumigatus extract (Greer Laboratories, Lenoir, NC, USA), R. oryzae extract, or were left unpulsed. The frequency of R. oryzae-reactive T cells was measured in PBMC populations 6–7 days after each stimulation at a T cell/monocyte ratio of 2:1 (1 × 10^6 effector cells: 5 × 10^5 target cells). Millipore Multi Screen HTS filter plates (Millipore, Billerica, MA, USA) were coated with IFN-γ capture antibody (Mabtech, Cincinnati, OH, USA) at a concentration of 10 μg/mL for 4 hr or overnight at 4°C. Plates were washed with PBS and blocked for 1 hr at 37°C. For development, the plates were washed in PBS/0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and incubated with biotinylated IFN-γ detection antibody (0.5 μg/mL; Mabtech, Cincinnati, OH, USA) for 2 hr at 37°C, followed by incubation with streptavidin-coupled alkaline phosphatase complex (Vectastain; Vector Laboratories, Burlingame, CA, USA) for 1 hr at room temperature, after which the spots were developed by incubation with 3-amino-9-ethylcarbazole substrate (Sigma) solution. IFN-γ SFCs were counted and evaluated with an automated plate reader system (Zellnet Consulting; Karl Zeiss, Oberkochen, Germany). To determine the population (or mechanism) responsible for the IFN-γ secretion, we used antibodies that could block specific killing pathways as previously described.31 Autologous monocytes were activated by overnight plastic adherence and pulsed with the relevant antigen (positive control) or with an irrelevant antigen (negative control) and then co-incubated with blocking antibodies against HLA class I and II, as well as MRs and TLRs (TLR2, TLR4, TLR6, and dectin-1) (Dako North America, CA, USA). Blocking of MHC class I and II was performed by incubation of T cells with these antibodies for 1 hr before plating for ELISpot assay.32 ELISpot plates were incubated and developed as described above. The human 4-color FluoroSpot Kit was used to measure for secretion of IFN-γ, IL-2, TNF-α, and granzyme B by Mucor-specific T cells (Immunospot, Cleveland, OH, USA). Cells were plated at 1e5 cells/well on a 4-color FluoroSpot, with the appropriate negative controls: media only, actin (JPT, Berlin, Germany), or monocytes only. Cells were plated at 1e5 cells/well on a 4-color FluoroSpot, with the appropriate positive controls: SEB (Sigma Aldrich, St. Louis, MO, USA) or mucor lysate with monocytes. FluoroSpot plates were developed according to the kit protocol and sent for unbiased spot counting to Immunospot.

Multi-Th17 Cytokine Analysis
Supernatants were harvested at 24 hr after the third stimulation/encounter with antigen and were temporarily stored at –80°C for batch processing. For comparison, samples were also obtained at the beginning of the culture period/during the first stimulation and were obtained from cells in the presence/absence of R. oryzae antigen. Samples were thawed and aliquoted into a Luminex plate in duplicate wells according to the manufacturer’s instructions (Millipore, Billerica, MA, USA). We used the Milliplex Map Human Th17 Premixed 25-Plex Magnetic Bead Panel to determine cytokine concentrations per sample, each of which was placed in duplicate wells and analyzed on the Bio-Plex MAGPIX Multiplex Reader (Bio-Rad) using Bio-Plex Manager software (Bio-Rad).
Statistical Analysis
Unless otherwise specified, data are presented as means ± SD. When relevant, T cell responses measured by ELISPOT and multi-cytokine ELISA were compared by Wilcoxon matched-pairs signed rank test (irrelevant antigen versus relevant antigen being tested). A p value <0.05 was considered to be significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.03.003.

AUTHORS CONTRIBUTIONS
Conceptualization, Investigation, Writing – Original Draft, P.C. and K.E.W.; Investigation and Formal Analysis, S.P., E.C., Y.H., S.B., N.A., and B.O.; Resources and Writing – Review & Editing, N.A. and D.P.K.; Conceptualization, Methodology, Resources, and Writing – Review & Editing, A.M.L. and T.W.; Conceptualization, Methodology, Writing – Original Draft, Writing – Review & Editing, Resources, and Funding Acquisition, C.M.R., C.M.B., and C.R.Y.C.

CONFLICTS OF INTEREST
C.R.Y.C. and C.M.B. are founding members of Mana Therapeutics. C.M.B. is on the scientific board of Viracyte and Marker, and is on the scientific board of Cell Medica. The other authors declare that there are no conflicts of interests.

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