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. The expanded populations included 17%–83% CD4$^+$ T cells that were specific for *R. oryzae* antigens. Besides interferon-γ (IFN-γ), these cells secreted IL-5, IL-10, IL-13, and tumor necrosis factor alpha (TNF-α), and recognized fungal antigens presented by HLA-II molecules rather than through nonspecific signaling. The method described herein is robust and reproducible, and could be used to generate adequate quantities of activated *R. oryzae*-specific T cells for clinical testing of safety and antifungal efficacy in patients with mucormycosis.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is the only curative option for many hematological malignancies that are refractory to standard chemotherapies and radiotherapies.[1] However, opportunistic infections are a major cause of morbidity and mortality posttransplant,[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,[5] and accounts for 8%–13% of all invasive fungal infections.[4,6] This frequency is increasing[7] 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
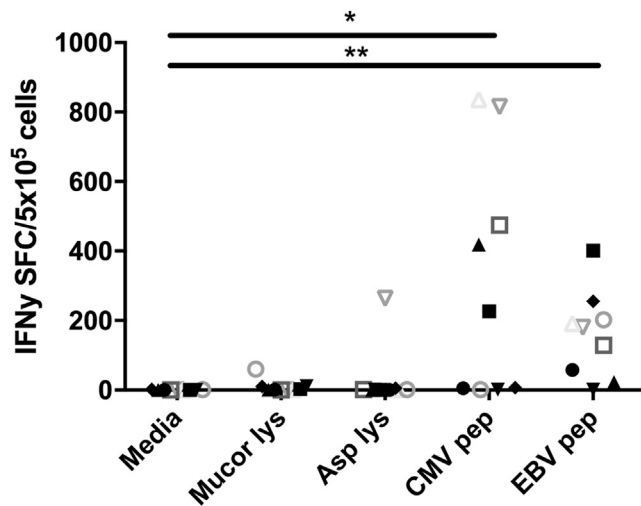
Figure 1. Frequency of Fungi-Reactive T Cells in Healthy Donor PBMCs

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.

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.[25,26] 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]

RESULTS

R. oryzae-Specific T Cell Responses Are Not Readily Detected in the Peripheral Blood of Most Healthy Donors

We screened nine healthy donors to evaluate the T cell responses against *R. oryzae* antigens and did not observe any appreciable changes in interferon-γ (IFN-γ) secretion after 48 hr of incubation of peripheral blood mononuclear cells (PBMCs) with *R. oryzae*-pulsed activated monocytes (mean 9.78 IFN-γ spot-forming cells [SFCs]/5 × 10^5 cells, range 0–60; n = 9; p = 0.1742; Figure 1). By contrast, distinct responses were noted against the CMV antigens pp65 and IE1 (mean 309 IFN-γ SFCs/5 × 10^5 cells, range 1.5–835; n = 9; p = 0.0039) and the EBV antigens EBNA1, EBNA3a, EBNA3b, EBNA 3c, BZLF1, and LMP2 (mean 160 IFN-γ SFCs/5 × 10^5 cells, range 1.5–401, n = 9; p = 0.0039). The lack of *R. oryzae*-specific T cell response was mirrored by the results of *A. fumigatus* screening in 8 of the 9 donors tested (mean 30.3 IFN-γ SFCs/5 × 10^5 cells, range 0–264; n = 9; p = n.s. [not significant]; Figure 1), even when the PBMCs were exposed to viable fungal cultures. To confirm that the responses to *R. oryzae* antigens are at extremely low levels, we harvested supernatants from

PBMCs (n = 4 donors) stimulated with *R. oryzae*-pulsed monocytes and compared them with unpulsed monocytes. Using a Luminex assay, we observed that the reactivity was not statistically significant (p = 0.05) above the negative control (median 0 for no antigen, range 0–0; median 12.885 for 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.[29]

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.[30,31] 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 CD45RO^{+} 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 × 10^5 cells, in IL-4/IL-7 was 107 IFN-γ SFCs/1 × 10^5 cells, in IL-7/IL-15 was 988 IFN-γ SFCs/1 × 10^5 cells, and for IL-15/IL-21 it was only 28 IFN-γ SFCs/1 × 10^5 cells, with background responses to unpulsed monocytes of 28, 7, 112, and 2.4 IFN-γ SFCs/1 × 10^5 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% CD45RO). All expanded T cell products showed specificity for *R. oryzae* antigens by ELISpot with a mean of 344 IFN-γ SFCs/1 × 10^5 cells (range 57–1,057; n = 10) compared with a mean
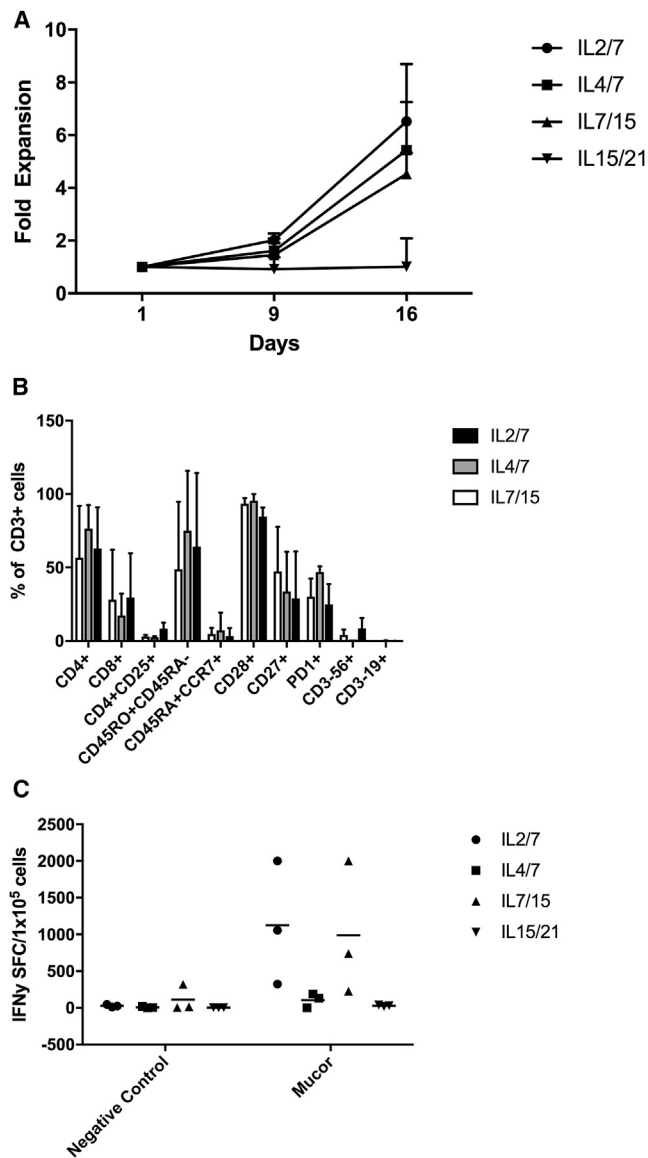
Figure 2. Optimization of Expansion Conditions

(A) Comparison of the expansion of T cell products derived from three healthy donors. T cells were stimulated with the cytokines 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 cytokines 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.
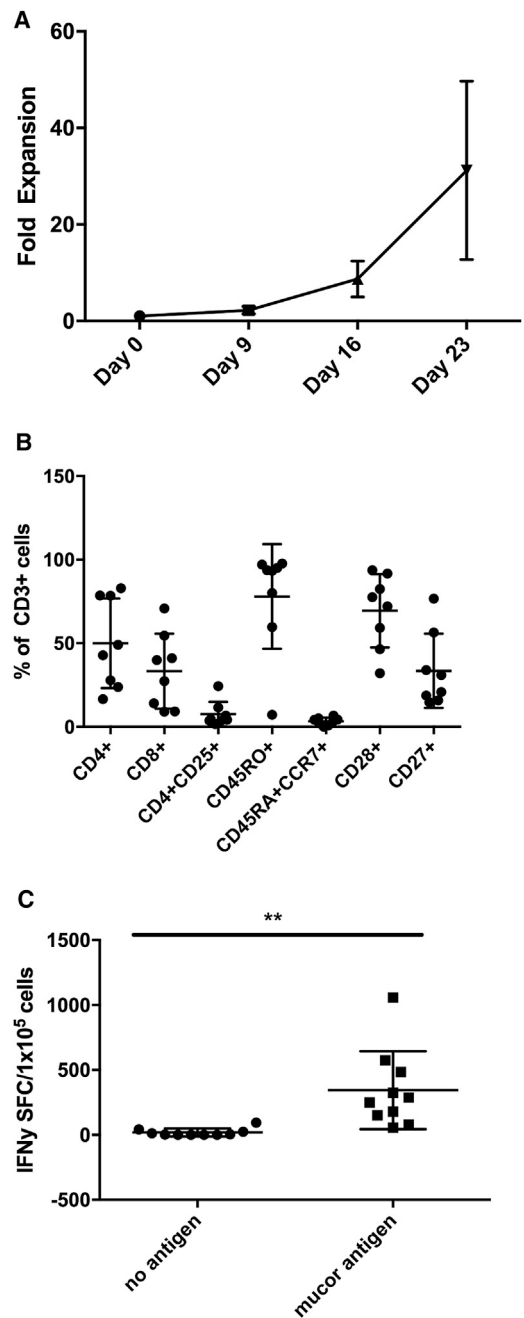

Figure 3. Characterization of *R. oryzae*-Specific T Cells Expanded with IL-2/IL-7

(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.
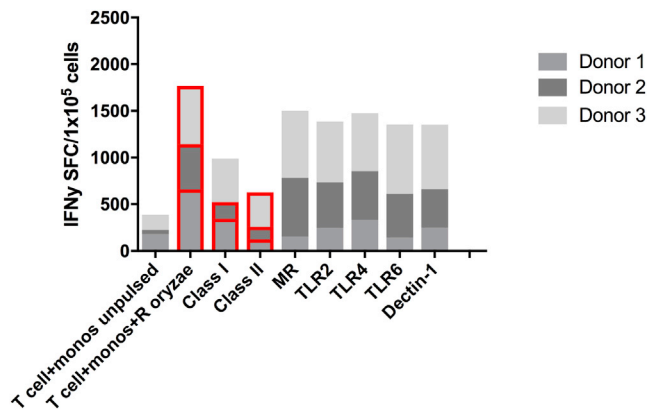
Figure 4. MHC Restriction of T Cells

T cells recognize antigens via class II, as shown by substantial decreases in the number of IFN-γ spots when class II was blocked (unblocked versus blocked) highlighted by red borders (n = 3).

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 antibodies that could block human leukocyte (HLA) class I and II recognition, as well as different PRRs (mannose receptor [MR], dectin 1, Toll-like receptor 2 [TLR2], TLR4, TLR6), in three healthy donors and measured their effects on IFN-γ secretion. As shown in Figure 4, blocking with class II antibodies decreased IFN-γ secretion by a mean of 65% (range 41%–81%; n = 3). Interestingly, in two of three donors tested, a more modest decrease in IFN-γ secretion was also seen after class I blocking (mean 44%), suggesting that for certain donors (and potentially certain HLA types), a CD8⁺ response may be important. In each of these donors, however, the CD4⁺ compartment still comprised the majority of the T cell population. No substantial changes in IFN-γ secretion were seen after blocking any of the five PRRs that were tested, compared with our control (T cell + monocytes + *R. oryzae* lysate).

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),
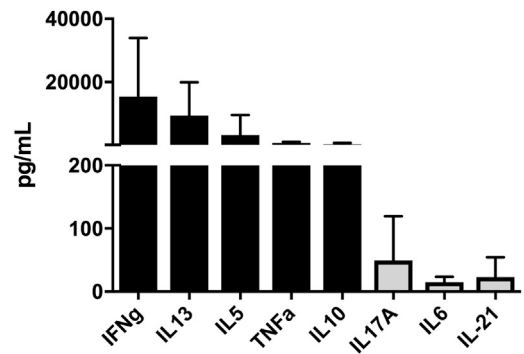

Figure 5. Cytokine Secretion of Expanded *R. oryzae*-Specific T Cells

Cytokine responses elicited from three *ex vivo* expanded *R. oryzae*-specific T cell products were analyzed after the third stimulation using a multiplex ELISA. Bars represent means, and error bars show SDs.

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 T helper 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

Table 1. Cytokine Expression of *Rhizopus oryzae*-Specific T Cells

Donor	IL-17F	GM-CSF	CCL20/MIP3a	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; MIP3a, macrophage inflammatory protein-3.

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),[33] (2) alloreactive T cells (IL-7/IL-15),[34] 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 leukophoresis 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 ($5 \times 10^6/m^2$ to $2 \times 10^7/m^2$).[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.[37] 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).[21] IFN-γ has long been associ-

ated 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,[39] whereas the early clearance of *R. oryzae* antigen relies on activation of eosinophils by IL-5.[40] 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,

BZLF1, and LMP2 PepMixes; JPT, Berlin, Germany), CMV peptides (a combination of pp65 and IE1 PepMixes; JPT, Berlin, Germany), and *Staphylococcus* enterotoxin B (SEB) or phytohemagglutinin as a positive control (Sigma-Aldrich, St. Louis, MO, USA) for 48 hr to allow antigen processing and efficient presentation by the APCs within the PBMCs (monocytes and B cells). ELISpot plates were developed as previously described.[17]

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),[32] 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 (Gemini 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, dialyzed 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 (1×10^5 effector cells: 5×10^4 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°, 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-ethyl-carbazole 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.[42] 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 advisory boards of Torque, Neximmune, and Cellectis. A.M.L. is a founding member of Viracyte. C.M.R. is a founding member of Viracyte and Marker, and is on the scientific advisory board of Cell Medica. The other authors declare that there are no conflicts of interests.

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REFERENCES

1. Pizzo, P.A., and Poplack, D.G. (2011). Principles and Practice of Pediatric Oncology (Wolters Kluwer/Lippincott Williams & Wilkins Health).

2. Leather, H.L., and Wingard, J.R. (2001). Infections following hematopoietic stem cell transplantation. Infect. Dis. Clin. North Am. 15, 483–520.

3. Thomas, E.D. (1999). Bone marrow transplantation: a review. Semin. Hematol. 36 (4 Suppl 7), 95–103.

4. Petrikkos, G., Skiada, A., Lortholary, O., Roilides, E., Walsh, T.J., and Kontoyiannis, D.P. (2012). Epidemiology and clinical manifestations of mucormycosis. Clin. Infect. Dis. 54 (Suppl 1), S23–S34.

5. Binder, U., Maurer, E., and Lass-Florl, C. (2014). Mucormycosis—from the pathogens to the disease. Clini. Microbiol. Infect. 20 (Suppl 6), 60–66.

6. Roden, M.M., Zaoutis, T.E., Buchanan, W.L., Knudsen, T.A., Sarkisova, T.A., Schaufele, R.L., Sein, M., Sein, T., Chiou, C.C., Chu, J.H., et al. (2005). Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin. Infect. Dis. 41, 634–653.

7. Bitar, D., Van Cauteren, D., Lanternier, F., Dannaoui, E., Che, D., Dromer, F., Desenclos, J.C., and Lortholary, O. (2009). Increasing incidence of zygomycosis (mucormycosis), France, 1997-2006. Emerg. Infect. Dis. 15, 1395–1401.

8. Kontoyiannis, D.P., Lionakis, M.S., Lewis, R.E., Chamilos, G., Healy, M., Perego, C., Safdar, A., Kantarjian, H., Champlin, R., Walsh, T.J., and Raad, I.I. (2005). Zygomycosis in a tertiary-care cancer center in the era of Aspergillus-active antifungal therapy: a case-control observational study of 27 recent cases. J. Infect. Dis. 191, 1350–1360.

9. Ambrosioni, J., Bouchuiguir-Wafa, K., and Garbino, J. (2010). Emerging invasive zygomycosis in a tertiary care center: epidemiology and associated risk factors. Int. J. Infect. Dis. 14 (Suppl 3), e100–e103.

10. Park, B.J., Pappas, P.G., Wannemuehler, K.A., Alexander, B.D., Anaissie, E.J., Andes, D.R., Baddley, J.W., Brown, J.M., Brumble, L.M., Freifeld, A.G., et al. (2011). Invasive non-Aspergillus mold infections in transplant recipients, United States, 2001-2006. Emerg. Infect. Dis. 17, 1855–1864.

11. Neofytos, D., Treadway, S., Ostrander, D., Alonso, C.D., Dierberg, K.L., Nussenblatt, V., Durand, C.M., Thompson, C.B., and Marr, K.A. (2013). Epidemiology, outcomes, and mortality predictors of invasive mold infections among transplant recipients: a 10-year, single-center experience. Transpl. Infect. Dis. 15, 233–242.

12. Lewis, R.E., Lortholary, O., Spellberg, B., Roilides, E., Kontoyiannis, D.P., and Walsh, T.J. (2012). How does antifungal pharmacology differ for mucormycosis versus aspergillosis? Clin. Infect. Dis. 54 (Suppl 1), S67–S72.

13. Marty, F.M., Ostrosky-Zeichner, L., Cornely, O.A., Mullane, K.M., Perfect, J.R., Thompson, G.R., 3rd, Alangaden, G.J., Brown, J.M., Fredricks, D.N., Heinz, W.J., et al.; VITAL and FungiScope Mucormycosis Investigators (2016). Isavuconazole treatment for mucormycosis: a single-arm open-label trial and case-control analysis. Lancet Infect. Dis. 16, 828–837.

14. Pagano, L., Caira, M., Candoni, A., Offidani, M., Fianchi, L., Martino, B., Pastore, D., Picardi, M., Bonini, A., Chierichini, A., et al. (2006). The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica 91, 1068–1075.

15. Lanternier, F., Sun, H.Y., Ribaud, P., Singh, N., Kontoyiannis, D.P., and Lortholary, O. (2012). Mucormycosis in organ and stem cell transplant recipients. Clin. Infect. Dis. 54, 1629–1636.

16. Fujita, Y., Rooney, C.M., and Heslop, H.E. (2008). Adoptive cellular immunotherapy for viral diseases. Bone Marrow Transplant. 41, 193–198.

17. Leen, A.M., Myers, G.D., Sili, U., Huls, M.H., Weiss, H., Leung, K.S., Carrum, G., Krance, R.A., Chang, C.C., Molldrem, J.J., et al. (2006). Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. Nat. Med. 12, 1160–1166.

18. Papadopoulou, A., Gerdemann, U., Katari, U.L., Tzannou, I., Liu, H., Martinez, C., Leung, K., Carrum, G., Gee, A.P., Vera, J.F., et al. (2014). Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci. Transl. Med. 6, 242ra83.

19. Cortez, K.J., Lyman, C.A., Kottilil, S., Kim, H.S., Roilides, E., Yang, J., Fullmer, B., Lempicki, R., and Walsh, T.J. (2006). Functional genomics of innate host defense molecules in normal human monocytes in response to Aspergillus fumigatus. Infect. Immun. 74, 2353–2365.

20. Roilides, E., Kontoyiannis, D.P., and Walsh, T.J. (2012). Host defenses against zygomycetes. Clin. Infect. Dis. 54 (Suppl 1), S61–S66.

21. Romani, L. (2011). Immunity to fungal infections. Nat. Rev. Immunol. 11, 275–288.

22. Potenza, L., Vallerini, D., Barozzi, P., Riva, G., Forghieri, F., Zanetti, E., Quadrelli, C., Candoni, A., Maertens, J., Rossi, G., et al. (2011). Mucorales-specific T cells emerge in the course of invasive mucormycosis and may be used as a surrogate diagnostic marker in high-risk patients. Blood 118, 5416–5419.

23. Potenza, L., Vallerini, D., Barozzi, P., Riva, G., Gilioli, A., Forghieri, F., Candoni, A., Cesaro, S., Quadrelli, C., Maertens, J., et al. (2016). Mucorales-specific T cells in patients with hematologic malignancies. PLoS ONE 11, e0149108.

24. Schmidt, S., Tramsen, L., Perkhofer, S., Lass-Flörl, C., Röger, F., Schubert, R., and Lehrnbecher, T. (2012). Characterization of the cellular immune responses to Rhizopus oryzae with potential impact on immunotherapeutic strategies in hematopoietic stem cell transplantation. J. Infect. Dis. 206, 135–139.

25. Tramsen, L., Schmidt, S., Boenig, H., Latgé, J.P., Lass-Flörl, C., Roeger, F., Seifried, E., Klingebiel, T., and Lehrnbecher, T. (2013). Clinical-scale generation of multi-specific anti-fungal T cells targeting Candida, Aspergillus and mucormycetes. Cytotherapy 15, 344–351.

26. Deo, S.S., Virassamy, B., Halliday, C., Clancy, L., Chen, S., Meyer, W., Sorrell, T.C., and Gottlieb, D.J. (2016). Stimulation with lysates of Aspergillus terreus, Candida krusei and Rhizopus oryzae maximizes cross-reactivity of anti-fungal T cells. Cytotherapy 18, 65–79.

27. Shoham, S., Magill, S.S., Merz, W.G., Gonzalez, C., Seibel, N., Buchanan, W.L., Knudsen, T.A., Sarkisova, T.A., and Walsh, T.J. (2010). Primary treatment of zygomycosis with liposomal amphotericin B: analysis of 28 cases. Med. Mycol. 48, 511–517.

28. Skiada, A., Pagano, L., Groll, A., Zimmerli, S., Dupont, B., Lagrou, K., Lass-Florl, C., Bouza, E., Klimko, N., Gaustad, P., et al.; European Confederation of Medical Mycology Working Group on Zygomycosis (2011). Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis between 2005 and 2007. Clin. Microbiol. Infect. 17, 1859–1867.

29. Khanna, N., Stuehler, C., Conrad, B., Lurati, S., Krappmann, S., Einsele, H., Berges, C., and Topp, M.S. (2011). Generation of a multipathogen-specific T-cell product for adoptive immunotherapy based on activation-dependent expression of CD154. Blood 118, 1121–1131.

30. Hanley, P.J., Melenhorst, J.J., Nikiforow, S., Scheinberg, P., Blaney, J.W., Demmler-Harrison, G., Cruz, C.R., Lam, S., Krance, R.A., Leung, K.S., et al. (2015). CMV-specific T cells generated from naïve T cells recognize atypical epitopes and may be protective in vivo. Sci. Transl. Med. 7, 285ra63.

31. Weber, G., Gerdemann, U., Caruana, I., Savoldo, B., Hensel, N.F., Rabin, K.R., Shpall, E.J., Melenhorst, J.J., Leen, A.M., Barrett, A.J., and Bollard, C.M. (2013). Generation of multi-leukemia antigen-specific T cells to enhance the graft-versus-leukemia effect after allogeneic stem cell transplant. Leukemia 27, 1538–1547.

32. Leen, A., Ratnayake, M., Foster, A., Heym, K., Ahmed, N., Rooney, C.M., and Gottschalk, S. (2007). Contact-activated monocytes: efficient antigen presenting cells for the stimulation of antigen-specific T cells. J. Immunother. 30, 96–107.

33. Gerdemann, U., Keirnan, J.M., Katari, U.L., Yanagisawa, R., Christin, A.S., Huye, L.E., Perna, S.K., Ennamuri, S., Gottschalk, S., Brenner, M.K., et al. (2012). Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. Mol. Ther. 20, 1622–1632.

34. Kaneko, S., Mastaglio, S., Bondanza, A., Ponzoni, M., Sanvito, F., Aldrighetti, L., Radrizzani, M., La Seta-Catamancio, S., Provasi, E., Mondino, A., et al. (2009). IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. Blood 113, 1006–1015.

35. Huarte, E., Fisher, J., Turk, M.J., Mellinger, D., Foster, C., Wolf, B., Meehan, K.R., Fadul, C.E., and Ernstoff, M.S. (2009). Ex vivo expansion of tumor specific lymphocytes with IL-15 and IL-21 for adoptive immunotherapy in melanoma. Cancer Lett. 285, 80–88.

36. Deo, S.S., and Gottlieb, D.J. (2015). Adoptive T-cell therapy for fungal infections in haematology patients. Clin. Transl. Immunology 4, e40.

37. Kumaresan, P.R., Manuri, P.R., Albert, N.D., Maiti, S., Singh, H., Mi, T., Roszik, J., Rabinovich, B., Olivares, S., Krishnamurthy, J., et al. (2014). Bioengineering T cells to target carbohydrate to treat opportunistic fungal infection. Proc. Natl. Acad. Sci. USA 111, 10660–10665.

38. Cunha, C., Di Ianni, M., Bozza, S., Giovannini, G., Zagarella, S., Zelante, T., D'Angelo, C., Pierini, A., Pitzurra, L., Falzetti, F., et al. (2010). Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. Blood 116, 5394–5402.

39. Singh, P., Taylor, S.F., Murali, R., Gomes, L.J., Kanthan, G.L., and Maloof, A.J. (2007). Disseminated mucormycosis and orbital ischaemia in combination immunosuppression with a tumour necrosis factor alpha inhibitor. Clin. Experiment. Ophthalmol. 35, 275–280.

40. Eddens, T., Elsegeiny, W., Nelson, M.P., Horne, W., Campfield, B.T., Steele, C., and Kolls, J.K. (2015). Eosinophils contribute to early clearance of Pneumocystis murina infection. J. Immunol. 195, 185–193.

41. Lefèvre, L., Authier, H., Stein, S., Majorel, C., Couderc, B., Dardenne, C., Eddine, M.A., Meunier, E., Bernad, J., Valentin, A., et al. (2015). LRH-1 mediates anti-inflammatory and antifungal phenotype of IL-13-activated macrophages through the PPARγ ligand synthesis. Nat. Commun. 6, 6801.

42. McLaughlin, L.P., Lang, H., Williams, E., Wright, K.E., Powell, A., Cruz, C.R., Colberg-Poley, A.M., Barese, C., Hanley, P.J., Bollard, C.M., and Keller, M.D. (2016). Human parainfluenza virus-3 can be targeted by rapidly ex vivo expanded T lymphocytes. Cytotherapy 18, 1515–1524.