MUCORALES-SPECIFIC T CELLS EMERGE IN THE COURSE OF INVASIVE MUCORMYCOSIS AND MAY BE USED AS A SURROGATE DIAGNOSTIC MARKER IN HIGH RISK PATIENTS.

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Short Title: Specific T cells to diagnose invasive mucormycosis

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Summary

Mucorales-specific T cells have been investigated in 28 hematologic patients during the course of their treatment. Three developed proven invasive mucormycosis (IM), 17 infections of known etiologies but other than IM, and 8 never showed fever upon the period of observation. The Mucorales-specific T cells may be detected only in patients with IM, at diagnosis and along the entire course of the IM, but neither before nor long time after the resolution of the infection. Such T cells produced predominantly interleukin-4, interferon-gamma (IFN-γ), interleukin-10, and to a lesser extent also interleukin-17, and belonged to either CD4+ or CD8+ subsets. The specific T cells producing IFN-γ were able to directly induce damage of Mucorales hyphae. None of the 25 patients without IM showed Mucorales-specific T cells. Specific T cells contribute to human immune responses against fungi of the order Mucorales and could be evaluated as a surrogate diagnostic marker of IM.

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Invasive Mucormycosis (IM), the second-most common cause of invasive mold infections in hematologic patients, shows mortality rates approaching 70% of affected individuals, because of difficulties in obtaining an early and undoubted diagnosis\textsuperscript{1-5}. Actually, a definitive diagnosis of IM relies exclusively on both histopathological demonstration and cultural isolation of the pathogen from the involved organs\textsuperscript{6}. However, obtaining tissue specimens in hematologic patients is too often hampered by the presence of several comorbidities, and histologically proven IM may fail to grow in culture in at least 1/3 of cases\textsuperscript{7}. Furthermore, neither serologic nor antigenic diagnostic methods exist and the use of polymerase chain reaction (PCR) has almost exclusively been limited to the identification and the discrimination of fungal species\textsuperscript{8,9}.

Adaptive immunity has been reported to play a crucial role in the defence of the host against fungi, at least in the case of invasive aspergillosis (IA) and invasive candidiasis\textsuperscript{10,11}, and the recognition and enumeration of antigen-specific T cells has been demonstrated a useful tool for the diagnosis of definite infectious diseases, in particular of either active or latent tuberculosis\textsuperscript{12}.

We have explored the possibility that Mucorales-specific T cells are elicited in patients with IM and that their detection may be of value in the diagnosis of active disease.
PATIENTS AND METHODS

Twenty-eight hematologic patients have been studied. Patients 1-3 had disseminated (pulmonary and splenic), tracheo-bronchial and cerebral proven IM, respectively (Figures S1, S2).

The antifungal treatments of the three patients with proven IM have been described in details in supplemental data.

The remaining 25 cases included 17 patients presenting infectious complications of proven etiology on the basis of cultural and/or histologic examinations, but other than IM, and 8 who have not developed infectious complications during the course of their induction chemotherapy. Patients' clinical characteristics have been reported in Table S1. Informed consent was obtained in accordance with the Declaration of Helsinki, and the study was approved by the University of Modena and Reggio Emilia Ethical Committee.

The enzyme linked immunospot (ELISpot) assay has been performed to detect either Mucorales- or Aspergillus-specific T cell, as reported\textsuperscript{13} and described in details in supplemental data, on 80 peripheral blood samples (range 2 to 6 per patients). Time points analysed were: in patient 1, the beginning of induction chemotherapy, 20 days before the pulmonary biopsy, at the histological and cultural identification of Rhizomucor pusillus infection, the beginning of consolidation chemotherapy, and 16 weeks after the resolution of the infection; in patient 2, the day of cultural and histologic demonstration of Rhizopus oryzae infection, and until death in four further occasions during the course of IM; in patient 3, the day of histologic and molecular demonstration of Absydia corymbifera infection, three occasions during the course of IM, and at the complete resolution of the infection. All the other patients were analysed at least twice during the course of their either infections or chemotherapeutic treatments (Table S1).

The phenotypic and functional characterization of Mucorales-specific T cells has been performed with the cytokine secretion assay (CSA) as already reported\textsuperscript{14} and described in details in supplemental data. Molecular studies, micromanipulation and single-cell PCR to identify Mucorales species (Figure S2) were performed as previously reported\textsuperscript{15} and described in details in
supplemental data. Anti-Mucorales activity of specific T cells was performed as reported in supplemental data.
RESULTS

Identification of Mucorales-specific T cells

In patient 1, the ELISpot resulted positive for the presence of Mucorales-specific T cells producing interleukin (IL)-10 in the second, third, fourth and fifth samples, and Mucorales-specific T cells producing interferon gamma (IFN-γ) in the second, third and fourth samples. In contrast, no Mucorales-specific T cells could be detected before the occurrence of the infection (at day +1 of induction chemotherapy) and long time after its resolution (day +238) (Figure 1A).

In patient 2 and 3, the ELISpot showed the sole presence of Mucorales-specific T cells producing IL-10 in the first sample (on the day of cultural and histologic demonstration of IM) in both patients; increasing numbers of Mucorales-specific T cells producing IFN-γ in the second, third and fourth samples in patient 2, and in the third and fourth samples in patient 3; the occurrence of Mucorales-specific T cells producing IL-4 in the fourth sample in patient 2 and in the third and fourth samples in patient 3. The last examination demonstrated the sole presence of Mucorales-specific T cells producing IL-10 in patient 2, close to death, and the absence of specific responses in patient 3, at the time of complete resolution of the infection (Figure 1B,C).

The differences between the median frequencies of Mucorales-specific T cells producing IL-10, IFN-γ and IL-4 did not result statistically significant in all the three patients (p = .3), not even when the results of the first two patients with more disseminated diseases were compared with those of the third patient, with a more limited infection (p = .5).

In the 25 control patients, the ELISpot never showed the presence of Mucorales-specific T cells. None of the analysed patients demonstrated the occurrence of Aspergillus-specific T cell at any time point (Table S1).

Phenotypic and Functional Characterization of Mucorales-specific T cells

In patients 1-3, Mucorales-specific T cells resulted: 1) predominantly CD8+ T cells (mean CD8+/CD4+ frequencies 3.62%/0.57%) of CM phenotype, producing IFN-γ; 2) predominantly CD8+ T cells (mean CD8+/CD4+ frequencies 4.35%/2.60%) of EM phenotype producing IL-4; 3)
either CD4+ or CD8+ T cells (mean CD4+/CD8+ frequencies 0.32%/0.26%), the former of either CM or EM phenotype, the latter mainly of CM, producing IL-10. *Mucorales*-specific T cells producing IL-17 were also detectable, being either CD4+ or CD8+ (mean frequency 0.44% and 0.56%, respectively), and exhibiting, predominantly, the CM phenotype (Figure 2A,B).

*Lytic Activity of Mucorales-specific T cells*

*Mucorales*-specific T cells from patients 1-3 were capable to induce direct damage to the hyphae of the two clinical isolates, similar to that of either PMNs or APCs. Only the combination of all the three cell types resulted in a significantly higher hyphal damage (p<0.05) (Figure 2C,D).
DISCUSSION

We have shown, for the first time, that Mucorales-specific T cells may occur during the infection course in patients with IM and exhibit direct antifungal activity, comparable, at least in vitro, to that of either PMNs or APCs. The contribution of T cells to host defences against these moulds could only be suspected, based on the enhanced fungicidal activity against Mucorales of polymorphonuclear leukocytes exposed to IFN-γ\(^\gamma\)\(^{16}\), but it has not yet been formally demonstrated.

The presence of Mucorales-specific T cells only during the course of IM, but neither before nor after the resolution of the infection in patients 1-3, and their absence in patients without infections or with infections other than IM, suggest that they are closely related to the occurrence of IM and may result a marker of overt disease. Of note, the presence of Mucorales-specific T cells has been demonstrated the only proof of IM in patient 1, largely before the obtainment of the biopsy. The lower frequencies of specific T cells in patient 3 seem to suggest that a more confined IM could be associated with responses of inferior magnitude. However, no statistically significant differences were observed between the median numbers of Mucorales-specific T cells in our three patients.

Unfortunately, as all the samples were collected either when the patients were undergoing antifungal treatment or after the drug withdrawal, no interaction between antifungal therapy and the occurrence of Mucorales-specific T cells could be determined in our study.

The cytokine production profile of Mucorales-specific T cells, in our study, is partially in line with what observed either in mice affected by IA or in human T cell clones stimulated with different Aspergillus antigens in vitro\(^{17-19}\). The demonstration that CD8+ Mucorales-specific T cells may produce either IL-4 or IL-10, predominantly in the late phase of the infection, is reminiscent of the type 2 cytokine shift of CD8+ lymphocytes, so far reported only in patients with the cavitary phase of tuberculosis and the late phase of human immunodeficiency virus infection\(^{20-21}\).

In conclusion, Mucorales-specific T cells emerge in the course of IM and contribute to the human immune responses against Mucorales. The detection of Mucorales-specific T cells may be evaluated as a surrogate diagnostic marker of IM.
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AUTHORSHIP CONTRIBUTIONS

LP and ML conceived, designed the study and wrote the manuscript; DV, PB, GiRi, GiRo, FR and MP made the ELISpot analysis, the CSA analysis, the XTT assays, histological examination, performed the molecular characterization of fungi and interpreted the data; AC, JM, FF, MoMo, MC, AP, MoMa, RM, FN provided well-characterized patient samples and critically revised the manuscript; CDG and RDA made the statistical analysis and interpreted the data; FC made the radiological studies and critically revised the manuscript.
DISCLOSURE OF CONFLICTS OF INTEREST

ML received research funds by Merck Sharp & Dohme and Gilead Sciences. ML serves in Advisory Boards for Merck Sharp & Dohme and Gilead Sciences, and received honoraria from these two pharmaceutical industries and from Pfizer and Nanogen. LP serves in an Advisory Board for Merck Sharp & Dohme. AC serves in Advisory Board for Merck Sharp & Dohme and received funds by Merck Sharp & Dohme, Gilead Sciences and Pfizer.

ML, LP and PB have applied for a European patent regarding clinical applications of the ELISpot assay for the diagnosis of Aspergillus infection [PCT: WO2008/075395A3, EP2094295, IT2007/000867]. ML, LP, DV, PB and FF have applied for an Italian patent regarding clinical applications of the ELISpot assay for the diagnosis of Mucorales infection (No. MI2010A002224). All the other authors have no conflicts of interest to declare. In particular they have neither employment, nor consultancy, including stock options in a start-up company, nor ownership interest in a publicly traded company, nor research funding. They received neither honoraria nor paid expert testimony. They have neither other potential financial relationship (e.g., holding a patent or receiving royalties), nor membership on another entity’s Board of Directors or its advisory committees.
REFERENCES


FIGURE LEGEND

Figure 1 A-C. Kinetics of Mucorales-specific T-cell responses, by IFN-γ, IL-10 and IL-4 ELISpot assay, in the three patients with invasive mucormycosis. A. Patient 1. B. Patient 2. C. Patient 3. Yellow columns represent the number of Mucorales-specific T cells producing interleukin 10 (IL-10). Blue columns represent the number of Mucorales-specific T cells producing interferon gamma (IFN-γ). Red columns represent the number of Mucorales-specific T cells producing interleukin 4 (IL-4). The dark grey background represents the T-cell responses in wells with phytohemagglutinin (PHA). Vertical axis shows the number of spot forming cells (SFCs) per million of peripheral blood mononuclear cells (PBMCs). Horizontal axis indicates the time in days from the beginning of induction chemotherapy.

Figure 2 A-D. Cytokine production profile and lytic activities of Mucorales-specific T cells.
A,B. The frequencies of Mucorales specific T cells producing IFNγ, IL-10, IL-4 or IL-17, either as EM (light grey) or CM (dark grey), are shown as mean % positive cells, computed over the three patients with IM. Results are expressed as percentages of either CD4+ T cells (A) or of CD8+ T cells (B). Mean frequencies of specific cytokine-producing T cells for individual patients are reported on each column, either as EM (□) or CM (O). EM = effector memory, CM = central memory.
C,D. Hyphal damage at 2 (C) and 22 (D) hours to Rhizomucor pusillus and Rhizopus oryzae induced by anti-Mucorales T cells (T), polymorphonuclear leukocytes (PMNs), and antigen-presenting cells (APCs), alone or in combination, deriving from patient 1 and patient 2 during the course of IM. E:T = effector/target cells ratios.
Figure 1
Figure 2
Mucorales-specific T cells emerge in the course of invasive mucormycosis and may be used as a surrogate diagnostic marker in high-risk patients

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