Calcineurin inhibitors impair neutrophil activity against *Aspergillus fumigatus* in allogeneic hematopoietic stem cell transplant recipients

Sébastien Imbert, MS,[a] Priscillia Bresler, MS,[b] Alexandre Boissonnas, PhD,[b] Lauraine Gauthier, PharmD,[a] Laëtitia Souchet, MD,[c] Madalina Uzunov, MD,[c] Véronique Leblond, MD, PhD,[b,c] Dominique Mazier, MD, PhD,[a,b] Stéphanie Nguyen, MD, PhD,[b,c] and Arnaud Fekkar, PharmD, PhD[a,b] *Paris, France*

Background: Neutrophils are key effectors against the widely distributed mold *Aspergillus fumigatus*, which is a major threat for immunocompromised patients, including allogeneic hematopoietic stem cell transplant (HSCT) recipients. Yet little is known about neutrophil activity over time after cell transplantation, especially regarding *A fumigatus*.

Objective: We aimed at assessing the activity of neutrophils on *A fumigatus* in allogeneic HSCT recipients at different posttransplantation time points.

Methods: We performed a longitudinal study involving 37 patients undergoing HSCT, drawing blood samples at engraftment and at 2, 6, and 10 months after the HSCT. Posttransplantation neutrophil activity in the recipients was compared with that of the respective donors. Neutrophil/*A fumigatus* coculture, flow cytometry, and video microscopy were used to assess neutrophil inhibition of fungal growth, cell/fungus interactions, reactive oxygen species production, major surface molecule expression, and neutrophil extracellular trap (NET) formation.

Results: The ability of neutrophils to interfere with *Aspergillus* species hyphal growth was impaired after HSCT. The administration of calcineurin inhibitors appeared to play an important role in this impairment. We also observed that post-HSCT neutrophils produced less NETs, which was correlated with increased fungal growth. Tapering immunosuppression led to the recuperation of inhibition capacity 10 months after HSCT.

Conclusion: In HSCT recipients neutrophil-driven innate immunity to fungi is altered in the early posttransplantation period (between recovery from neutropenia and up to 6 months). This alteration is at least partly related to administration of calcineurin inhibitors and diminution of NETs production. (J Allergy Clin Immunol 2016;138:860-8.)

Key words: *Innate immunity, calcineurin inhibitor, invasive fungal infection, immune reconstitution, transplantation*

Aspergillus fumigatus is the main causative agent of invasive aspergillosis, which is a major threat to immunocompromised patients, including hematopoietic stem cell transplant (HSCT) recipients. Polymorphonuclear neutrophil cells (called neutrophils hereafter) are key effectors against fungal infection. In contrast to monocytes and macrophages, which phagocytose resting conidia, neutrophils are also able to act against germinating conidia and hyphae through a trapping mechanism, giving them particular importance against *Aspergillus* species.[1,2] However, only a few studies have provided data on the behavior of neutrophils after HSCT, and how these cells regain their basic functions after transplantation is unknown.

The choice of immunosuppressors for the prevention of graft-versus-host disease (GvHD) can vary depending on the conditioning regimen, type of donor, and type of graft. The most common prophylaxis strategy associates a calcineurin inhibitor (mainly cyclosporine A) with methotrexate, anti-thymoglobulin antibodies, or mycophenolate mofetil.[3] Despite those immunosuppressive drugs, acute GvHD occurs in approximately 40% to 60% of patients undergoing HSCT and requires administration of corticosteroids.[3] The action of cyclosporine, which is essentially known for its effect on adaptive immunity, is mediated by inhibition of calcineurin, a calcium-dependent phosphatase. Particularly, calcineurin activates the NFAT transcription factors, leading to transcription and production of many T-cell effector cytokines, such as IL-2.[4-6] Very few data exist concerning its potential role on innate immunity. Patients receiving cyclosporine run an increased risk of viral, bacterial, or fungal infection. Among these pathogens, the mold *Aspergillus* species is particularly dangerous, causing high morbidity and mortality. The administration of cyclosporine or other recognized T-cell immunosuppressors is included in the host criteria for probable invasive fungal disease, as established by the European Organisation for Research and Treatment of Cancer and the Mycoses Study Group.[7]

Thus, for the present study, we aimed to evaluate neutrophil function and activity toward *Aspergillus* species in HSCT recipients over time. We assessed neutrophil activity, including fungal growth inhibition, oxidative burst, surface molecule expression, and neutrophil extracellular trap (NET) production, after different stimuli.

From [a]AP-HP, Groupe Hospitalier La Pitié-Salpêtrière, Service de Parasitologie Mycologie; [b]Sorbonne Universités, UPMC Univ Paris 06, INSERM U1135, CNRS ERL 8255, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris); and [c]AP-HP, Groupe Hospitalier La Pitié-Salpêtrière, Service d'Hématologie.

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Corresponding author: Arnaud Fekkar, PharmD, PhD, Laboratoire de Parasitologie-Mycologie, Pavillon Laveran, Hôpital de La Pitié-Salpêtrière, Boulevard de l'Hôpital, 75013 Paris, France. E-mail: arnaud.fekkar@aphp.fr.

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Abbreviations used
CD62L: CD62 ligand
GvHD: Graft-versus-host disease
HSCT: Hematopoietic stem cell transplant
NET: Neutrophil extracellular trap
ROS: Reactive oxygen species
TLR: Toll-like receptor

METHODS

Patients and donors

The present longitudinal study involved 37 patients who received a related (36/37) or unrelated (1/37) allogeneic HSCT for a malignant hemopathy in La Pitié-Salpêtrière Hospital, Paris, France. Blood samples were collected from the recipients at engraftment and then at 2, 6, and 10 months after transplantation and were compared with samples taken from their respective donors. This noninterventional study was approved by the local ethics committee (CPP Ile de France IV). Signed informed consent forms were obtained from all donors and recipients.

Neutrophil isolation

Neutrophils were isolated by using the dextran-Ficoll method. Briefly, whole fresh blood was mixed with an equivalent volume of 2.0% dextran solution (Sigma-Aldrich, St Louis, Mo) in normal saline, and red blood cells were allowed to settle for 40 minutes at 4°C. Then the leukocyte-rich supernatant was submitted to Ficoll (Eurobio, Courtaboeuf, France) centrifuge separation for 30 minutes at 700*g* and 4°C. After elimination of remaining red blood cells, neutrophils in the pellet were recovered in RPMI medium and tested immediately.

A fumigatus strain

An *A fumigatus* sensu stricto strain isolated from clinical samples in La Pitié-Salpêtrière Hospital was used. The strain was maintained on Sabouraud with chloramphenicol and gentamicin agar tubes at 37°C for 5 to 7 days. Conidia were harvested with PBS containing 0.05% Tween 20, washed 3 times, and suspended in PBS and counted.

Aspergillus species growth inhibition

Black, 96-well, clear-bottom plates (Greiner, Frickenhausen, Germany) were seeded with 1500 conidia per well in RPMI medium containing 1% FCS and allowed to germinate for 7 hours at 37°C. After this growth period, *Aspergillus* species measuring approximately 15 to 20 μm can be considered either as germinating conidia or as small hyphae. The medium was then changed to RPMI without FCS, and the isolated neutrophils were added to the wells at different effector/target ratios in triplicate. In other experiments neutrophils were added directly to resting conidia. The plates were incubated overnight at 37°C with 5% CO_2 and then washed with purified water. Uvitex (1% wt/vol; Polysciences, Warrington, Pa), a fluorescent marker of chitin (similar to calcofluor, which has already been used to assess fungal biomass[8]) was added to the final dilution. Finally, plates were read with a FlexStation analyzer (Molecular Devices, Sunnyvale, Calif), with excitation at 350 nm and emission at 435 nm. For a given effector/target ratio, fungal growth was determined as the ratio of the fluorescence intensity of the well containing neutrophils mixed with *Aspergillus* species to that of the well containing *Aspergillus* species only. The percentage of inhibition was defined as 100 minus the percentage of fungal growth.

Surface molecule expression of neutrophils

Five-hundred-microliter whole-blood samples were stimulated with either 10^6 *Aspergillus* species conidia (resting or germinating), 5 ng/mL bacterial LPS (Sigma-Aldrich), or PBS as a control for 45 minutes at 37°C. Neutrophils were stained with an anti-human CD11b (or integrin alpha M) antibody (Dako, Glostrup, Denmark), an anti-human CD62 ligand (CD62L; or L-selectin) antibody (Becton Dickinson, San Jose, Calif), and/or an anti-human CD66 (or carcinoembryonic antigen) antibody (Becton Dickinson) before cytometric analysis. Toll-like receptor (TLR) and dectin expression were assessed by using anti-TLR2, anti-TLR4, and anti–Dectin-1 antibodies (R&D Systems, Minneapolis, Minn). Cytometry was performed on a Gallios flow cytometer, and results were analyzed with Kaluza software (Beckman Coulter, Fullerton, Calif).

TABLE I. Characteristics of patients included in the study and their conditioning regimens

Mean age (y [minimum-maximum])	44 (20-69)
Male/female sex	19/18
Disease, no. (%)	
Acute myeloid leukemia	15 (40.5)
Lymphoma	9 (24.3)
Lymphoid leukemia	5 (13.5)
Myelofibrosis	3 (8.1)
Multiple myeloma	2 (5.4)
Others	3 (8.1)
Type of donor, no. (%)	
Matched related donor	32 (86.5)
Matched unrelated donor	1 (2.7)
Haploidentical related donor	4 (10.8)
Type of graft, no. (%)	
Bone marrow	14 (37.8)
Peripheral stem cell	23 (62.2)
Conditioning regimen, no. (%)	
Busulfan-based RIC	20 (54.1)
Thiotepa-based RIC	3 (8.1)
MAC*	11 (29.7)
Fludarabine and cyclophosphamide	3 (8.1)

MAC, Myeloablative conditioning; *RIC*, reduced-intensity conditioning.
*Myeloablative conditioning involved either total body irradiation and cyclophosphamide (n = 6) or busulfan and cyclophosphamide (n = 5).

Measurement of neutrophil oxidative burst

Neutrophils contained in 500 μL of heparinized whole-blood samples were incubated with hydroethidine (final concentration, 1.5 μg/mL; Sigma-Aldrich) for 15 minutes at 37°C and then stimulated with either 10^6 *Aspergillus* species conidia (resting or germinating), 5 ng/mL LPS, or PBS as a control for 45 minutes at 37°C. Then phorbol 12-myristate 13-acetate (final concentration, 10 μmol/L) or PBS was added for 5 minutes. Samples were then analyzed by using flow cytometry.

Video microscopy and NET formation assessment

Interactions between *Aspergillus* species and neutrophils were visualized with a Zeiss Axio Microscope (Carl Zeiss, Oberkochen, Germany). After 3 hours of coculture, SYTOX Green (Life Technologies, Grand Island, NY) was added to each well at a final dilution of 1:5000. Images were processed, and hyphal length was measured with ImageJ software (National Institutes of Health, Bethesda, Md). Quantification of NET formation was evaluated, as previously described,[9] with ImageJ software.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif) was used for statistical analyses.

RESULTS

Patients' characteristics

Patient data are presented in Table I. Mean donor and recipient ages were 47 years (range, 19-67 years) and 44 years (range,