

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

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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;■■■:■■■-■■■.)

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.

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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 700g 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% CO₂ 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 biomass⁸) 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 10⁶ *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,

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).

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).

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 10⁶ *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,

TABLE II. Characteristics of blood samples and immunosuppressive drug regimens 1, 2, 6, and 10 months after transplantation

	Month 1	Month 2	Month 6	Month 10
No. of samples tested	35	29	18	10
Neutrophil count (cells/ μ L), mean/median (minimum-maximum)	2680/2190 (390-6860)	2710/2260 (330-6320)	3480/2680 (880-7050)	3280/3350 (1020-6010)
Neutrophils (%), mean/median (minimum-maximum)	52.48/52 (7-78)	61.5/56 (29-92)	59.7/59 (30-89)	56.6/60 (19-74)
Time to engraftment (d), mean/median (minimum-maximum)	21.7/20 (13-37)	64.5/63 (54-85)	186/183 (173-203)	313/318 (268-336)
Immunosuppressive regimen (% of patients)				
Cyclosporine	28 (80)	25 (86.2)	1 (5.6)	1 (10)
Tacrolimus	3 (8.6)	1 (3.4)	2 (11.1)	0
Mycophenolate mofetil	0	1 (3.4)	0	0
Cyclosporine plus mycophenolate mofetil	3 (8.6)	1 (3.4)	1 (5.6)	1 (10)
Tacrolimus plus mycophenolate mofetil	1 (2.9)	2 (6.9)	0	0
Sirolimus	0	1 (3.4)	2 (11.1)	1 (10)
Corticosteroid	0	10 (34.5)	5 (27.8)	1 (10)
None	0	0	9 (50)	7 (70)

20-69 years), respectively. There were 23 male and 13 female donors and 19 male and 18 female recipients. No significant differences were observed concerning age ($P = .25$, Student test) or sex ratio ($P = .39$, χ^2 test). The main single indication for HSCT was acute myeloid leukemia (40.5% of patients), but lymphoproliferative disorders (ie, lymphoma, lymphoid leukemia, and myeloma) collectively accounted for 43% of cases. Other diseases included primary myelofibrosis and myelodysplastic syndrome.

The majority of patients (86.5%) underwent transplantation with a matched related donor. Conditioning regimens were mainly busulfan based, with reduced-intensity conditioning in 54.1% and myeloablative conditioning in 29.7%. All patients received GvHD prophylaxis involving a calcineurin inhibitor-based regimen plus other drugs depending on the type of graft and conditioning.

Neutrophils were collected from patients in the first month (recovery from neutropenia) and at 2, 6, and 10 months after HSCT. Recovery from neutropenia ("recovery" hereafter) was defined as the day on which the neutrophil count became greater than 500/mm³ (although for 1 patient we found *a posteriori* that this value was not reached). At recovery (median, 20 days), all patients were receiving a calcineurin inhibitor, usually cyclosporine (88.6%). No patients had corticosteroid therapy. Because of patient death or loss to follow-up, 10-month samples were available for only 10 patients. The characteristics of blood samples and immunosuppressive drug regimens at 1, 2, 6, and 10 months after HSCT are presented in Table II. Only donor-derived cells were detectable for all included recipients beyond approximately 2 months after transplantation (data not shown).

Neutrophil surface molecule expression after HSCT

Surface expression of CD11b (also known as integrin α M), CD62L (or L-selectin), and CD66 was evaluated at the basal level and after stimulation by resting/germinating conidia or LPS. In association with the β_2 -integrin (or CD18), CD11b forms the heterodimeric integrin macrophage 1 antigen, which is involved in leukocyte adhesion and migration. CD11b is expressed at the neutrophil surface after degranulation because it is contained in secondary and tertiary neutrophil granules. CD62L is involved in transient tethering of the neutrophils to the endothelial surface.

The shedding of CD62L marks an activation of the neutrophils. CD66 is specific to secondary granules.¹⁰ Surface expression of the major pattern recognition receptors TLR2, TLR4, and Dectin-1 were also assessed by using flow cytometry. Neutrophils were found to be activated in only a limited manner by resting conidia, but they were strongly activated by germinating conidia, as evidenced by an increase in CD11b and CD66 expression and a decrease in CD62L expression (Fig 1, A, and data not shown). As tested, LPS induced high activation. However, there were no observed differences over time. It is also noteworthy that no differences were observed between donors and patients during recovery, except as concerns the expression of CD66, which was higher after the graft (Fig 1, A and B). In comparison with donors, the expression of TLR4 and dectin did not change, but TLR2 expression diminished slightly in recipients during recovery (Fig 1, C).

Neutrophil reactive oxygen species production after HSCT

Because it is a major antipathogen mechanism of neutrophils, the production of reactive oxygen species (ROS) was analyzed at a basal level and after exposure to resting/germinating conidia or LPS. Resting conidia caused moderate ROS production, whereas germinating conidia led to an important oxidative burst (Fig 1, D and E). The level of ROS production was highly dependent on the stimulus ($P < .0001$, 2-way ANOVA), but it remained similar between recipients and their donors and stable over time ($P = .96$).

Neutrophil inhibition of *Aspergillus* species hyphae growth

Samples were available from 23 donors and 33 recipients at recovery to test *Aspergillus* species hyphae growth inhibition by neutrophils. This test set also permitted 19 paired sample comparisons. Unpaired (Fig 2, A) and paired (Fig 2, B) analysis showed a highly statistically significant decrease in the ability of neutrophils to hamper fungal growth during recovery compared with healthy donors.

Interestingly, there were no differences concerning the ability of neutrophils to inhibit the development of resting conidia

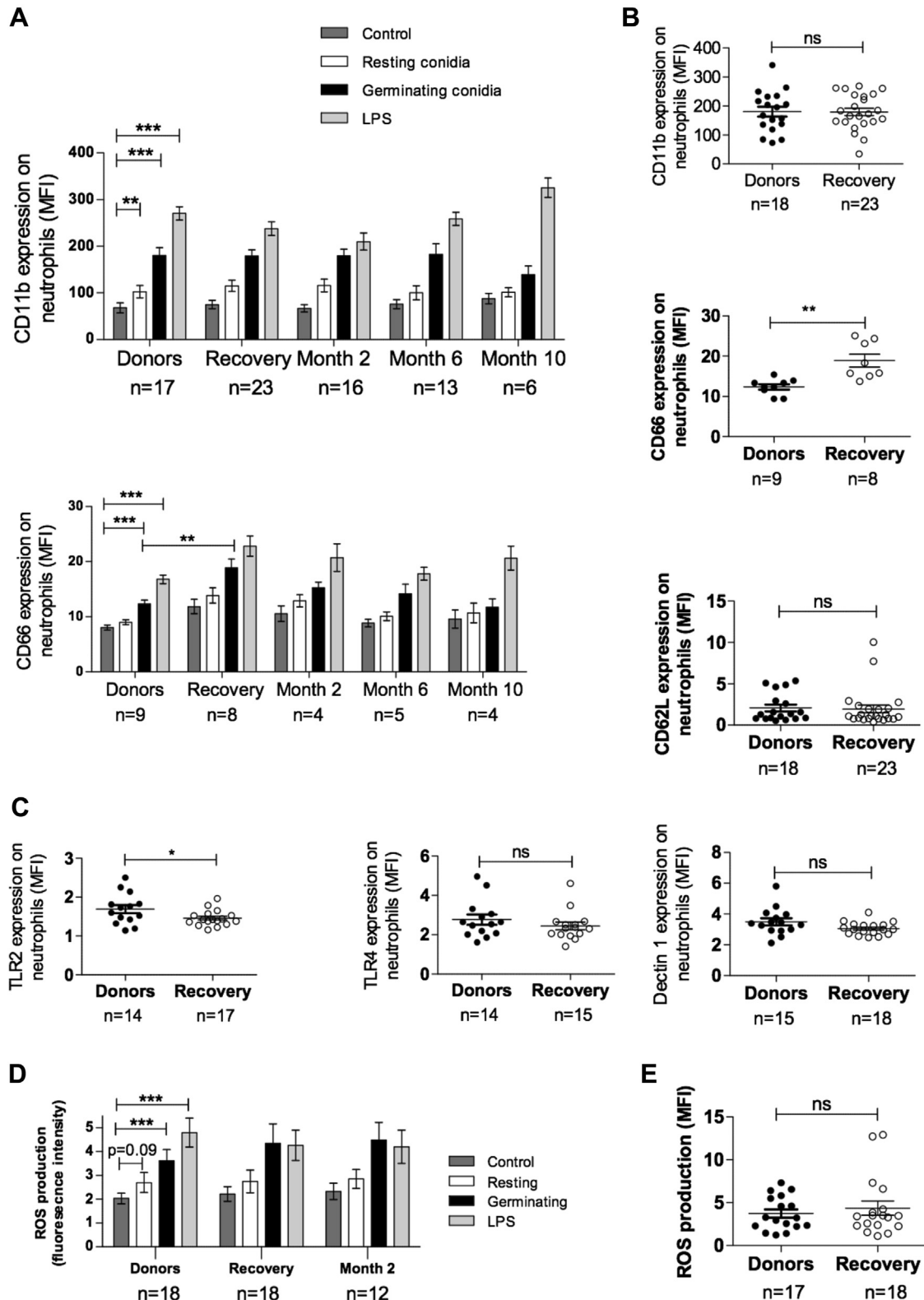


FIG 1. Postengraftment evolution of surface molecule expression and ROS production by neutrophils. **A**, Expression of CD11b and CD66, as determined by using flow cytometry. Neutrophils were collected at different time points after HSCT and stimulated with resting or germinating *Aspergillus* species conidia or LPS. Bars represent mean fluorescence intensity (MFI), with whiskers for the SEM. **B**, Expression of CD11b, CD66, and CD62L on neutrophils sampled from donors or recovering patients who underwent HSCT and stimulated by germinating *Aspergillus* species conidia. Long horizontal bars indicate MFI, with short bars for the SEM. **C**, Surface expression of TLR2, TLR4, and Dectin-1 on neutrophils sampled from

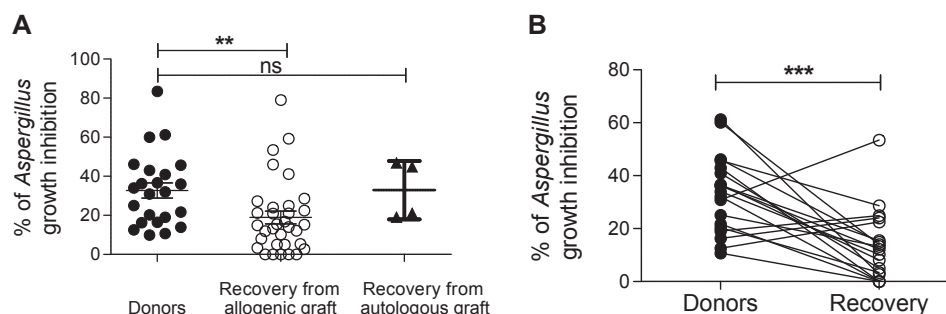


FIG 2. Ability of neutrophils to hamper *Aspergillus* species hyphae growth is impaired during recovery from neutropenia. Isolated neutrophils sampled from donors or allogeneic or autologous HSCT recipients during recovery were incubated with germinating conidia in a 96-well plate for 15 hours at 37°C with 5% CO₂. After washing, fungal growth was assessed by using a fluorescent probe. Growth inhibition was calculated as 1 – (Ratio of the fluorescence intensity of the well with neutrophils and *Aspergillus* species to that of the well with *Aspergillus* species only) and expressed as a percentage. **A**, Comparison of inhibition between neutrophils from 23 donors and 33 allogeneic and 4 autologous graft recipients at a ratio of 16 neutrophils for 1 germinating conidia. Long horizontal bars indicate means, with short bars for SEMs. **B**, Paired comparison between neutrophils sampled from 19 HSCT recipients and their respective donors. ** $P < .005$ and *** $P < .001$. ns, Not significant.

(data not shown). Of note, no correlations were observed between the period between engraftment and sampling on the one hand and the percentage of inhibition on the other. There was also no link between the absolute neutrophil count at the time of sampling and the ability of neutrophils to inhibit *Aspergillus* species growth (data not shown).

Effect of calcineurin inhibitors on neutrophil impairment of *Aspergillus* species hyphal growth

During the study, some patients in recovery had neutrophils that were as effective as those of control subjects at inhibiting *Aspergillus* species growth. This was not related to time to engraftment, type of graft, type of conditioning, or absolute neutrophil count in blood. However, interestingly, we found that these patients had low plasma calcineurin inhibitor trough concentrations. Indeed, these treatments are difficult to balance and therefore are regularly monitored. Considering this, we compared neutrophils of patients with low plasma calcineurin inhibitor trough concentrations (ie, <120 ng/mL for cyclosporine and <6 ng/mL for tacrolimus) with those of patients with normal trough concentrations (ie, >150 and >10 ng/mL, respectively) for *Aspergillus* species inhibition and found that numbers of the latter were significantly greater in patients with low plasma concentrations (Fig 3, A). Moreover, Pearson correlation analysis showed a certain link between the percentage of inhibition and the plasma calcineurin inhibitor trough level ($r = -0.39$, $P < .05$; Fig 3, B). To strengthen the hypothesis that the observed effect was due to a pharmacologic effect and not a generic effect of recovery, which is a very complex phenomenon, we performed growth inhibition tests using neutrophils from patients recovering from autologous stem cell transplantation, in which calcineurin inhibitors are not

administered. In this setting we found no defects in the ability of neutrophils to impair *Aspergillus* species growth (Fig 2, A).

We also performed *in vitro* experiments to further assess the hypothesis that calcineurin inhibitors were correlated with reduced neutrophil inhibition. When blood sampled from healthy donors was incubated with cyclosporine at 37°C for 2 hours, the subsequently isolated neutrophils showed diminished activity in terms of *Aspergillus* species growth inhibition in comparison with untreated control subjects (Fig 4). Thus cyclosporine appears to impair neutrophil activity against *A. fumigatus* hyphae.

Alterations to NET production in the early post-HSCT period

Neutrophils can exert their function through different mechanisms, including phagocytosis, ROS production, degranulation, and NET production. As discussed above, neither ROS production nor degranulation (investigated by CD11b and CD66 surface expression) were altered in the present study. Neutrophils cannot phagocytose hyphae because of their size and support adhesion, but it has been shown that they produce NETs in contact with *Aspergillus* species, consequently inhibiting its growth.^{11,12} Thus we investigated the production of NETs by neutrophils and their effects on fungal growth in both patients undergoing HSCT and healthy donors.

Neutrophils were cocultured with *Aspergillus* species for 3 hours, and NET production was visualized with SYTOX Green. In accordance with the results presented above, *Aspergillus* species growth, as assessed by hyphal length measurement during the 3-hour culture, was greater in patients than in control subjects; inhibition of *Aspergillus* species growth requires contact between the fungus and neutrophils (Fig 5, A). The DNA area of the

donors or recovering patients who underwent HSCT. Long horizontal bars indicate MFI, with short bars for the SEM. **D**, ROS production by neutrophils according to different stimuli at different time points after HSCT. Bars represent MFI, with whiskers for the SEM. **E**, ROS production by neutrophils sampled from donors or recovering patients who underwent HSCT and stimulated by germinating *Aspergillus* species conidia. Long horizontal bars indicate means, with short bars for SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

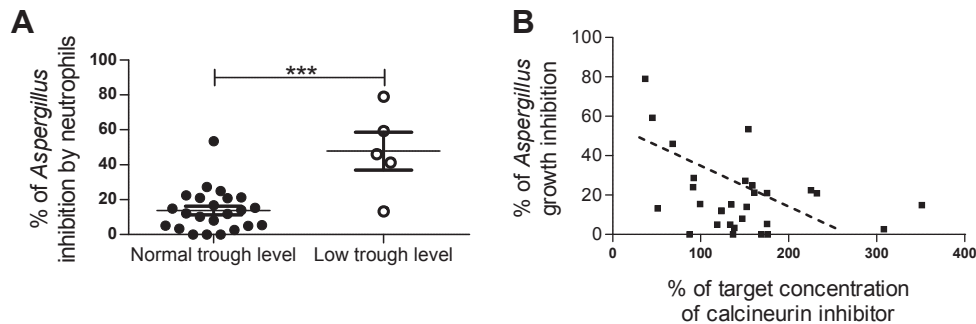


FIG 3. Calcineurin inhibitors reduce the ability of neutrophils to inhibit *Aspergillus* species hyphal growth. **A**, Neutrophils sampled from patients with normal calcineurin inhibitor trough levels (ie, >150 ng/mL for cyclosporine and >10 ng/mL for tacrolimus) are less capable of inhibiting *Aspergillus* species growth than those retrieved from patients with low trough levels (ie, <120 ng/mL for cyclosporine and <6 ng/mL for tacrolimus). Long horizontal bars indicate means, with short bars for SEMs. **B**, Correlation (Pearson test) between the percentage of inhibition of *Aspergillus* species by neutrophils (y-axis) and the percentage of target plasma calcineurin inhibitor trough level (x-axis). Target concentration was defined as 150 ng/mL for cyclosporine and 10 ng/mL for tacrolimus. Analysis included 26 patients receiving cyclosporine and 3 patients receiving tacrolimus. Concentrations were determined the day of or the day before neutrophil sampling, except for 2 patients for whom concentration values had been determined 2 days before sampling and 1 patient for whom concentration values had been determined 5 days before sampling. *** $P < .001$.

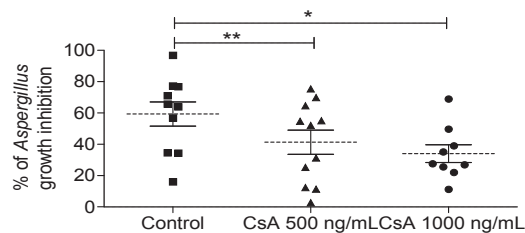


FIG 4. A calcineurin inhibitor diminishes the ability of neutrophils to inhibit *Aspergillus* species growth. Blood collected from healthy donors was incubated for 2 hours at 37°C with cyclosporine (CsA; final concentrations of 500 and 1000 ng/mL) or equivalent dimethyl sulfoxide vehicle as a control. Subsequently, isolated neutrophils were used for an *Aspergillus* species growth inhibition assay. Neutrophils preincubated with cyclosporine showed decreased fungal inhibition compared with untreated control cells. Long dashed bars indicate means, with short bars for SEMs. * $P < .01$ and ** $P < .005$.

SYTOX-positive cells was measured, as previously described,⁹ for NET quantification. Control subjects had a greater number of SYTOX-positive cells than did patients (Fig 5, B). Thus the results indicated that neutrophils of patients produce less NETs than those of control subjects (Fig 5, C-E).

Evolution of neutrophil inhibition of *Aspergillus* species growth and restoration of inhibition with cessation of immunosuppressive therapy

The evaluation of neutrophil action against *Aspergillus* species showed significant variation over time (Fig 6). Because of their general and previously reported effect on immunity against *Aspergillus* species,^{13,14} corticosteroid administration increased impairment of neutrophil activity against hyphae. Importantly, in the 10 patients studied 10 months after HSCT and for whom immunosuppressive therapies were stopped or considerably reduced, the percentage of *Aspergillus* species growth inhibition was restored to the control (donor) level. These results indicate (1) that the effect of calcineurin inhibitors can add to other immunosuppressive effects and (2) that these effects are not permanent, at least when treatment is stopped or decreased within some number of months.

DISCUSSION

The few studies that have focused on the behavior of neutrophils after HSCT provide a range of results suggesting moderate to no alterations in oxidative burst or antimicrobial activity.¹⁵ For the present study, we aimed at evaluating neutrophil function in HSCT recipients compared with that in healthy donors as concerns the major human pathogen mold *Aspergillus* species. We found that oxidative burst and surface molecule expression at basal levels and after stimulation did not vary significantly over time, with the exception of CD66, which was more highly expressed during recovery, and TLR2, which was slightly less expressed. However, our results show that the recovery period is associated with a dramatic decrease in the ability of neutrophils to inhibit *Aspergillus* species hyphae growth and that the use of calcineurin inhibitors might play a large role in this impairment. It is well known that HSCT recipients are particularly at risk for invasive aspergillosis, not only during the neutropenia period but also after engraftment. However, it should be acknowledged that neutrophil recovery is not the most at-risk period because antifungal prophylaxis is now widely used and, furthermore, that the use of a calcineurin inhibitor is probably not singly sufficient to trigger invasive aspergillosis, although cases of aspergillosis in patients receiving cyclosporine have been reported.¹⁶ Nevertheless, aspergillosis breakthrough in patients receiving antifungals is not rare,^{17,18} and the assessment of the ability of neutrophils to correctly impair (or not) *Aspergillus* species growth could be used to evaluate the risk of invasive aspergillosis in at-risk patients and thus contribute to reducing fungal risk. Nonetheless, calcineurin inhibitors do appear to add to other acquired or potential innate immune deficiencies that together favor the appearance of fungal disease, which can occur any time after HSCT. Indeed, approximately 20% of invasive mold infections are diagnosed early (<40 days after HSCT), 40% late (between 40 and 100 days after HSCT), and 40% very late (>100 days after HSCT).¹⁹ Whether this defect favors the occurrence of invasive aspergillosis and adds to genetic disorders (eg, TLR4²⁰ or IL-1β²¹) will need to be assessed in further studies.

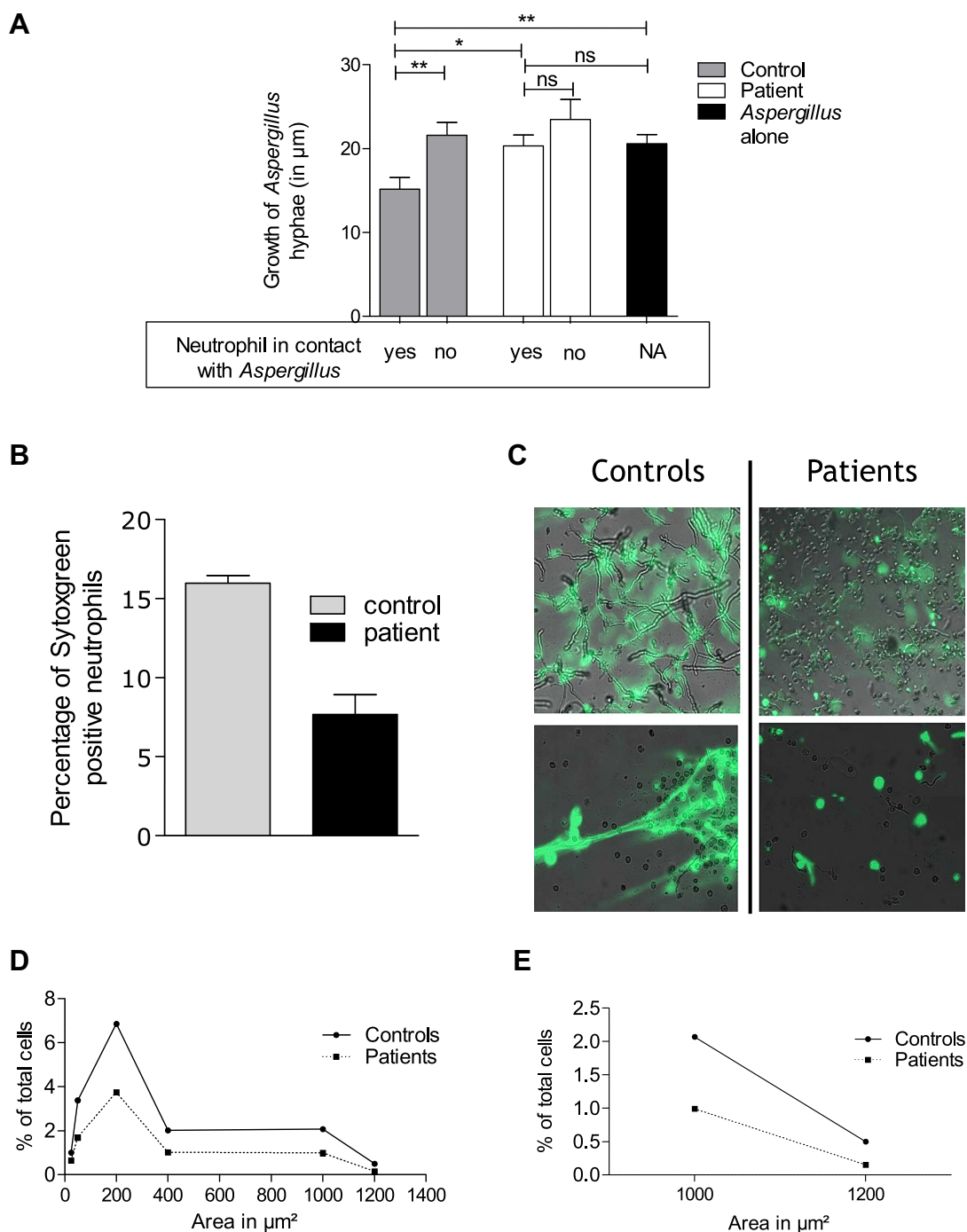


FIG 5. Neutrophil death and NET formation are decreased during recovery in allogeneic HSCT recipients. Neutrophils sampled from patients who underwent HSCT during recovery or from healthy donors were incubated with germinating *Aspergillus* species conidia for 3 hours at 37°C with 5% CO₂ in a 96-well plate placed on a Zeiss Axio Microscope. Pictures were taken every minute. SYTOX Green was added in each well at a final dilution of 1:5000 at the end of the experiment. Images were processed, and hyphal lengths were measured with ImageJ software. Quantification of NET formation was evaluated, as previously described by Papayannopoulos et al,⁹ with ImageJ software. **A**, Growth of *Aspergillus* species hyphae (in micrometers) after 3 hours of culture with or without neutrophils sampled from patients who underwent HSCT or control subjects. Length was measured for hyphae in contact with neutrophils and for those that were not. Inhibition of *Aspergillus* species growth (in micrometers) by neutrophils requires contact and is altered in patients who underwent HSCT. Results are representative of experiments with 4 patients and 4 control subjects. Bars represent means, with whiskers for the SEM. **B**, The death rate (assessed by SYTOX Green) in neutrophils sampled from control subjects is higher than that in neutrophils collected from patients. **C**, Images of 2 independent experiments showing the typical aspect of NETosis in control subjects, whereas the neutrophils of patients show aspects evocative of apoptosis/necrosis. **D-E**, NET formation assessed by measurement of an area of greater than 400 μm² is higher with control neutrophils than with neutrophils from patients who underwent HSCT, as assessed by the repartition of the SYTOX Green-positive neutrophils according to the area of the signal (Fig 5, D) and the percentage of the SYTOX Green-positive neutrophils that underwent NETosis (ie, with a signal area >400 μm²; Fig 5, E). **P* < .01 and ***P* < .005. ns, Not significant.

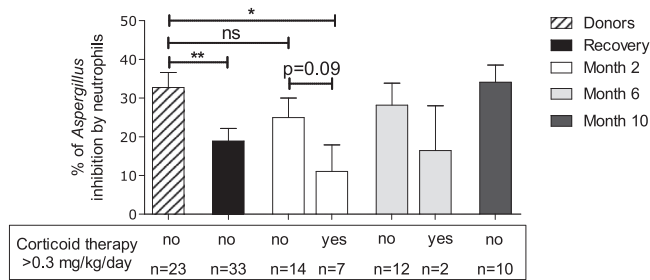


FIG 6. Evolution of *Aspergillus* species hyphae growth inhibition by neutrophils sampled from HSCT recipients. Growth inhibition is impaired during recovery but restored after 10 months. Adjunction of corticosteroid in patients who had GvHD is related to a trend toward a reduction of the ability of neutrophils to inhibit fungal growth. Bars represent means, with whiskers for the SEM. * $P < .01$ and ** $P < .005$. ns, Not significant.

For years, calcineurin inhibitors have been thought to exert their activity almost exclusively by targeting lymphocytes. However, over the past few years, a growing body of evidence suggests that they also have important effects on innate immunity. In mouse models of invasive aspergillosis, the administration of cyclosporine has been shown to shorten survival.^{22,23} However, nonconcordant results have been reported in other animal studies, and it remains unclear whether cyclosporine alone is sufficient to favor the development of invasive aspergillosis.^{23,24} More recently, Greenblatt et al⁵ used *in vitro* and murine models to show that calcineurin regulates neutrophil immunity against the yeast *Candida albicans*. They reported that mice treated with cyclosporine were more highly susceptible to disseminated *Candida* species infection than were control subjects and that both calcineurin-deficient neutrophils and cyclosporine-treated neutrophils showed an impaired response to *Candida* species. More recently, Tourneur et al²⁵ showed that cyclosporine impaired human neutrophil function, but their patients were kidney transplant recipients who were also receiving corticosteroid therapy, a known modifier of neutrophil function.

Neutrophils can act against extracellular pathogens by releasing NETs, which are composed of a DNA web containing histones and proteins with antimicrobial activity, such as lactoferrin or elastase.²⁶ McCormick et al¹¹ reported that NETs were able to reduce the polar growth of *Aspergillus* hyphae. Interestingly, a recent study reported that cyclosporine reduced IL-8–induced NET formation.²⁷

Calcineurin inhibitors modulate several pathways, including NFATc, nuclear factor κ B, and activator protein 1.^{28,29} Thus they not only inhibit the phosphatase activity of calcineurin but also the peptidyl-prolyl *cis-trans* isomerase activity of their respective receptor, immunophilin. It would be of interest to test the range of inhibitors that target the complex calcineurin pathway²⁹ to uncover the mechanism by which NETs are processed. It would also be of great interest to know whether targeting one or the other of these pathways would inhibit only alloreactive memory/effector T cells, therefore preventing rejection or GvHD, without impairing the innate immunity necessary for infection prevention. For now, there are not enough data to accurately describe the mechanism by which calcineurin inhibitors limit NET formation. Additionally, published results often appear contradictory. Gupta et al²⁷ found that NETosis was reduced dramatically by cyclosporine and ascomycin (an analogue of tacrolimus), whereas

rapamycin, which targets the mammalian target of rapamycin, had only a small effect. Inversely, McInturff et al³⁰ found that NETosis was inhibited by rapamycin but not tacrolimus. However, we underline that those 2 studies used different agents to induce NETosis: IL-8 for the former and LPS for the latter. In our work we found that neutrophils sampled from patients in recovery were less efficient at inhibiting *Aspergillus* species growth than those collected from healthy donors. ROS production was not impaired during recovery, and no correlation was observed between the percentage of inhibition and the level of oxidative burst after stimulation by germinating conidia (data not shown). In contrast, Stuehler et al³¹ reported recently that the ROS production of neutrophils collected from patients undergoing HSCT and stimulated by *Aspergillus* species was altered 30 days after the graft. Interestingly, however, their results also indicate that the percentage of fungal damage mediated by neutrophils was decreased in patients undergoing HSCT compared with control subjects, even in patients with normal ROS production.³¹ In accordance with that, it has been shown that cyclosporine does not impair ROS production.²³ Finally, in our microscopy experiments we observed that in cocultures with *Aspergillus* species, neutrophils sampled from patients in recovery produced less NETs than those sampled from healthy donors, with a higher rate of fungal growth. However, rather than a specific effect on NETosis, it seems that this diminished NET production is more generally a reflection of a lower neutrophil death rate. The implication of calcineurin in cell death was first reported long ago,³² and the enhancement of cell survival with the use of calcineurin inhibitors has been observed with different cell types.^{33,34}

In conclusion, this study exposes a previously unknown deficiency in the antifungal response of innate immunity in grafted patients. In HSCT recipients neutrophil-driven immunity against *A. fumigatus* is altered during the first month after transplantation; administration of calcineurin inhibitors plays an important role in this impairment, which is in stark contrast to the classical view of these inhibitors as affecting only adaptive immunity. The specific pathway by which these drugs alter neutrophil antifungal response and NET formation must now be investigated in further studies, with the particular goal of enabling the development of more specific therapeutic alternatives capable of inhibiting alloreactive effector T cells without impairing the basic functions of innate immunity cells. Attaining this goal will be challenging, but it is also vital for improving care in both HSCT and solid organ transplants.

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Key messages

- The ability of neutrophils to impair *Aspergillus* hyphal growth is altered during recovery in allogeneic HSCT recipients.
- The administration of calcineurin inhibitors seems to play an important role in this impairment, which is in stark contrast to the classical view of these inhibitors as affecting only adaptive immunity.

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