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

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ABSTRACT

Background: Neutrophils are key effectors against the widely distributed mold *Aspergillus fumigatus*, which is a major threat for immunocompromised patients including allogenic 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 allogenic HSCT recipients at different post-transplant time points.

Methods: We performed a longitudinal study involving 37 HSCT patients, drawing blood samples at engraftment and at two, six and ten months after the HSCT. Post-transplant neutrophil activity in the recipients was compared to that of the respective donors. Neutrophil/ *Aspergillus* co-culture, 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 traps (NETs) formation.

Results: The ability of neutrophils to interfere with *Aspergillus* 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 post-HSCT.

Conclusion: In HSCT recipients, neutrophil-driven innate immunity to fungi is altered in the early post-transplant period (between recovery from neutropenia and up to 6 months). This alteration is at least partly related to the administration of calcineurin inhibitors and the diminution of NETs production.

KEY MESSAGES

The ability of neutrophils to impair *Aspergillus* hyphal growth is altered during recovery in allogenic hematopoietic stem cell transplant recipients.

The administration of calcineurin inhibitors seems to play an important role in this impairment, in stark contrast to the classical view of these inhibitors as affecting only adaptive immunity.

CAPSULE SUMMARY

In allogenic hematopoietic stem cell transplant recipients, fungal innate immunity driven by neutrophils is altered during the first months of the graft, in relation with the use of calcineurin inhibitors.

KEYWORDS

Innate immunity; Calcineurin inhibitor; Invasive fungal infection; Immune reconstitution; Transplantation

ABBREVIATIONS

HSCT: hematopoietic stem cell transplant

GvHD: graft versus host disease

NETs: neutrophil extracellular traps

ROS: reactive oxygen species

INTRODUCTION

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 phagocyte resting conidia, neutrophils are also able to act against germinating conidia and hyphae through a trapping mechanism, giving them particular importance against *Aspergillus* [1, 2]. However, only a few studies have provided data on the behavior of neutrophils following HSCT, and additionally, how these cells regain their basic functions after the transplant is unknown.

The choice of immunosuppressors for the prevention of graft versus host disease (GvHD) can vary depending on the conditioning regimen, the type of donor and the type of graft. The most common prophylaxis strategy associates a calcineurin inhibitor (mainly cyclosporine A) with methotrexate (MTX), anti-thymoglobulin antibodies (ATG) or mycophenolate mofetil (MMF) [3]. Despite those immunosuppressive drugs, acute GvHD occurs in approximately 40-60% of HSCT patients [3] and requires the administration of corticosteroids [3]. The action of cyclosporine A, 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 the transcription and production of many T-cell effector cytokines such as interleukin 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* 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 (EORTC) and the Mycoses Study Group (MSG) [7].

Thus, for the present study, we aimed at evaluating neutrophil function and activity toward *Aspergillus* in HSCT recipients over time. We assessed neutrophil activity, including fungal

growth inhibition, oxidative burst, surface molecule expression, and neutrophil extracellular traps (NETs) production, following different stimuli.

MATERIALS AND 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, then at two, six and ten months post-transplant, and were compared to samples taken from their respective donors. This non-interventional study was approved by the local ethics committee (CPP Ile de France IV). Signed informed consent was obtained from all donors and recipients.

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

***Aspergillus* 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° for 5-7 days. Conidia were harvested with phosphate-buffered-saline (PBS) containing 0.05% Tween 20, washed three times and suspended in PBS and counted.

***Aspergillus* growth inhibition.** Black, 96-well clear-bottom plates (Greiner) were seeded with 1,500 conidia per well in RPMI medium containing 1% fetal calf serum (FCS) and allowed to germinate for 7 hours at 37°C. After this growth period, *Aspergillus* measuring ap-

proximately 15-20 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 others experiments, neutrophils were added directly to resting conidia. The plates were incubated overnight at 37°C with 5% CO_2 then washed with purified water. Uvitex (1% w/v), 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 using a Flexstation analyzer with excitation at 350 nm and emission at 435 nm. For a given effector:target ratio, the fungal growth was determined as the ratio of the fluorescence intensity of the well containing neutrophils mixed with *Aspergillus* to that of the well containing *Aspergillus* only. The percentage of inhibition was defined as 100 minus the percent of fungal growth.

Surface molecule expression of neutrophils. Five-hundred-microliter whole-blood samples were stimulated with either 10^6 *Aspergillus* conidia (resting or germinating), 5 ng/mL bacterial lipopolysaccharide (LPS) (Sigma Aldrich) or PBS as control for 45 minutes at 37°C. Neutrophils were stained with an anti-human CD11b (or integrin alpha M) antibody (Dako), an anti-human CD62L (or L-selectin) antibody (Becton Dickinson) and/or an anti-human CD66 (or carcinoembryonic antigen) antibody (Becton Dickinson) before cytometry analysis. TLR and dectin expression were assessed using anti-TLR2, anti-TLR4 and anti-dectin-1 antibodies (RD Systems). Cytometry was performed on a Gallios flow cytometer and results were analyzed using Kaluza software (Beckman Coulter).

Measurement of neutrophil oxidative burst. Neutrophils contained in 500 µL heparinized whole-blood samples were incubated with hydroethidine (Sigma Aldrich) (final concentration 1.5 µg/mL) for 15 minutes at 37°C, then stimulated with either 10^6 *Aspergillus* conidia (resting or germinating), 5 ng/mL LPS or PBS as control for 45 minutes at 37°C. Then PMA (final

concentration: 10 µM) or PBS was added for 5 minutes. Samples were then analyzed by flow cytometry.

Video microscopy and NETs formation assessment. Interactions between *Aspergillus* and neutrophils were visualized using a Zeiss Axio Microscope (Carl Zeiss, Germany). After 3 hours of co-culture, Sytox green (Life Technologies) was added to each well at a final dilution of 1/5000. Images were processed and hyphal length was measured using ImageJ software. Quantification of NETs formation was evaluated as previously described [9] with ImageJ.

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

RESULTS.

Patient characteristics.

Patient data are presented in Table 1. Mean donor and recipient ages were 47 (range 19-67) and 44 (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=0.25 by Student test) or sex ratio (p=0.39 by Chi-square test). The main single indication for HSCT was acute myeloid leukemia (40.5 % of patients) but lymphoproliferative disorders (i.e. 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%) were transplanted with a matched related donor. Conditioning regimens were mainly busulfan-based with reduced intensity (54.1%) or myeloablative (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 two, six and ten months post-HSCT. Recovery from neutropenia ("recovery" hereafter) was

defined as the day where the neutrophil count became >500/mm3 (although for one 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. Due to patient death or loss to follow-up, 10-month samples were available for only 10 patients. The characteristics of blood samples and immunosuppressive drugs regimens at one, two, six and ten months post-HSCT are presented in Table 2. Only donor-derived cells were detectable for all included recipients after approximatively two months post-transplant (data not shown).

Neutrophil surface molecule expression after HSCT

The surface expression of CD11b (also known as integrin alpha M), CD62L (or L-selectin) and CD66 was evaluated at the basal level and following stimulation by resting/germinating conidia or LPS. In association with the β_2 integrin (or CD18), CD11b forms the heterodimeric integrin macrophage-1 antigen involved in the adhesion and migration of leukocytes. CD11b is expressed at the surface of neutrophils after degranulation as 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 TLR-2, TLR-4 and dectin-1 were also assessed by 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 (Figure 1a and data not shown). As tested, LPS induced high activation. There were however 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 (Figure 1 a-b). In compari-

son with the donors, the expression of TLR4 and dectin did not change but TLR2 diminished slightly in recipients during recovery (Figure 1c).

Neutrophil reactive oxygen species production after HSCT.

As it is a major anti-pathogen mechanism of neutrophils, the production of reactive oxygen species (ROS) was analyzed at a basal level and following exposition to resting/germinating conidia or LPS. Resting conidia caused a moderate production of ROS while germinating conidia led to an important oxidative burst (Figure 1 d-e). The level of ROS production was highly dependent on the stimulus ($p<0.0001$ by 2-way ANOVA), but it remained similar between recipients and their donors, and stable over time ($p=0.96$).

Neutrophil inhibition of *Aspergillus* hyphae growth.

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

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

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

During the study, some patients in recovery had neutrophils that were as effective as controls at inhibiting *Aspergillus* growth. This was not related to time to engraftment, type of graft, type of conditioning, or absolute neutrophil count in blood. But interestingly, we found that these patients had low plasma calcineurin inhibitor trough concentrations. Indeed, these treatments are difficult to balance and therefore regularly monitored. Considering this, we compared the neutrophils of patients with low plasma calcineurin inhibitor trough concentrations (i.e. <120 ng/mL for cyclosporine and <6 ng/mL for tacrolimus) to those of patients with normal trough concentrations (i.e. >150 ng/mL and >10 ng/mL) for *Aspergillus* inhibition and found that this latter was significantly greater in patients with low plasma concentrations (Figure 3a). Moreover, Pearson correlation analysis showed a certain link between the percentage of inhibition and the plasma calcineurin inhibitor trough level ($r = -0.39$; $p<0.05$) (Figure 3b). To strengthen the hypothesis that the observed effect was due to a pharmacological 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, where calcineurin inhibitors are not administered. In this setting, we found no defects in the ability of neutrophils to impair *Aspergillus* growth (Figure 2a).

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* growth inhibition, in comparison to untreated controls (Figure 4). Cyclosporine thus appears to impair neutrophil activity against *A. fumigatus* hyphae.

Alterations to NETs production in early post-HSCT period.

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

Neutrophils were co-cultured with *Aspergillus* for three hours and NETs production was visualized using Sytox green. In accordance with the results presented above, *Aspergillus* growth as assessed by hyphal length measurement during the three-hour culture was greater in patients than in controls; inhibition of *Aspergillus* growth requires contact between the fungus and neutrophils (Figure 5a). The DNA area of the Sytox-positive cells was measured as previously described [9] for NETs quantification. Controls had a greater number of Sytox-positive cells than patients did (Figure 5b). The results thus indicated that neutrophils of patients produce less NETs than those of controls (Figure 5c-e).

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

The evaluation of neutrophil action against *Aspergillus* showed significant variations over time (Figure 6). Due to their general and previously reported effect on immunity against *Aspergillus* [13, 14], the administration of corticoids increased the impairment of neutrophil activity against hyphae. Importantly, in the 10 patients studied 10 months after the HSCT and for whom immunosuppressive therapies were stopped or considerably reduced, the percentage of *Aspergillus* growth inhibition was restored to the control (donor) level. These results indicate first that the effect of calcineurin inhibitors may add to other immunosuppressive effects, and

second, 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 following 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 to that in healthy donors as concerns the major human pathogen mold *Aspergillus*. We found that oxidative burst and surface molecule expression at basal levels and following stimulation did not vary significantly over time, with the exceptions 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* hyphae growth, and that the use of calcineurin inhibitors may 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. It should be acknowledged however that neutrophil recovery is not the most at-risk period since antifungal prophylaxis is now widely used, and furthermore that the use of a calcineurin inhibitor is probable 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* growth could be used to evaluate the risk of invasive aspergillosis in at-risk patients and thus contribute to reducing the 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 (e.g. TLR-4 [20] or IL-1β [21]) will need to be assessed in further studies.

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, non-concordant results have been reported in other animal studies and it remains unclear if cyclosporine alone is sufficient to favor the development of invasive aspergillosis [23, 24]. More recently, Greenblatt *et al* used *in vitro* and murine models to show that calcineurin regulates neutrophil immunity against the yeast *Candida albicans* [5]. They reported that mice treated with cyclosporine were more highly susceptible to disseminated *Candida* infection than were controls and that both calcineurin deficient neutrophils and cyclosporine treated neutrophils showed impaired response toward *Candida*. More recently, Tourneur *et al* showed that cyclosporine impaired human neutrophil function, but their patients were kidney transplant recipients who were also receiving corticoid therapy, a known modifier of neutrophil function [25].

Neutrophils can act against extracellular pathogens by releasing neutrophil extracellular traps (NETs), which are composed of a DNA web containing histones and proteins with antimicrobial activity, such as lactoferrin or elastase [26]. McCormick *et al* reported that NETs were able to reduce the polar growth of *Aspergillus* hyphae [11]. Interestingly, a recent study reported that cyclosporine reduced interleukin-8-induced NETs formation [27].

Calcineurin inhibitors modulate several pathways, including NFATc, NF-κB or AP-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 called immunophilin. It would be of interest to test the range of inhibitors that target the complex calcineurin pathway[29] 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 is not enough data to accurately describe the mechanism by which calcineurin inhibitors limit NETs formation. Additionally, published results often appear contradictory. Gupta *et al* found that NETosis was reduced dramatically by cyclosporine and ascomycin (an analogue of tacrolimus,) while rapamycin, which targets the mammalian target of rapamycin (mTOR), had only a small effect [27]. Inversely, McInturff *et al* found that NETosis was inhibited by rapamycin but not by tacrolimus [30]. We underline however that those two studies used different agents to induce NETosis: interleukin-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* 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 following stimulation by germinating conidia (data not shown). In contrast, Stuehler *et al* reported recently that the ROS production of neutrophils collected from HSCT patients and stimulated by *Aspergillus* was altered 30 days after the graft [31]. Interestingly however, their results also indicate that the percentage of fungal damage mediated by neutrophils was decreased in HSCT patients compared to controls, 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 co-cultures with *Aspergillus,* 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 ef-

fect 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 cellular types [33, 34].

CONCLUSION.

This study exposes a previously unknown deficiency in the antifungal response of innate immunity of grafted patients. In hematopoietic stem cell transplant recipients, neutrophil-driven immunity against *Aspergillus fumigatus* is altered during the first month post-transplant; administration of calcineurin inhibitors plays an important role in this impairment, 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 NETs 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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POTENTIAL CONFLICTS OF INTEREST.

None for all authors.

CONTRIBUTIONS.

SI performed experiments, PB performed experiments, AB designed and performed microscopic experiments and participated in writing the paper, LG performed experiments; LS, MU

and VL participated in the design of the study and the inclusion of patients, DM participated in scientific discussions and writing of the paper, SNG participated in the design of the study, scientific discussions, inclusion of patients and writing of the paper, AF designed the study, performed experiments and wrote the paper.

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Table 1: Characteristics of patients included in the study and their conditioning regimens

RIC: reduced-intensity conditioning; MAC: myeloablative conditioning

493 [a] Myeloablative conditioning involved either total body irradiation and cyclophosphamide

494 (n=6) or busulfan and cyclophosphamide (n=5)

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Mean age in years (minimum-maximum)		44 (20-69)
Male/female sex		19/18
Disease: n (%)	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	Matched related donor	32 (86.5)
	Matched unrelated donor	1 (2.7)
	Haploidentical related donor	4 (10.8)
Type of graft: n (%)	Bone marrow	14 (37.8)
	Peripheral stem cell	23 (62.2)
Conditioning regimen: n (%)	Busulfan-based RIC	20 (54.1)
	Thiotepa-based RIC	3 (8.1)
	MAC[a]	11 (29.7)
	Fludarabine and cyclophosphamide	3 (8.1)

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504 Table 2: Characteristics of blood samples and immunosuppressive drugs regimens 1, 2, 6, and

505 10 months post-transplant.

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		Month 1	Month 2	Month 6	Month 10
Number of samples tested		35	29	18	10
Neutrophil count: mean / median (cells/µL) [minimum-maximum]		2680 / 2190 [390-6860]	2710 / 2260 [330-6320]	3480 / 2680 [880-7050]	3280 / 3350 [1020-6010]
% of 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: mean / median [minimum-maximum] (days)		21.7 / 20 [13-37]	64.5 / 63 [54-85]	186 / 183 [173-203]	313 / 318 [268-336]
Immuno-suppressive 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 + mycophenolate mofetil	3 (8.6)	1 (3.4)	1 (5.6)	1 (10)
	Tacrolimus + mycophenolate mofetil	1 (2.9)	2 (6.9)	0	0
	Sirolimus	0	1 (3.4)	2 (11.1)	1 (10)
	Corticoid	0	10 (34.5)	5 (27.8)	1 (10)
	None	0	0	9 (50)	7 (70)

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515 **<u>Figure 1</u>: Post-engraftment evolution of surface molecule expression and reactive oxy-**

516 **gen species production by neutrophils**.

1a: Expression of CD11b and CD66 by flow cytometry. Neutrophils were collected at different time-points after HSCT and stimulated with resting or germinating *Aspergillus* conidia, or lipopolysaccharide (LPS). Bars represent mean fluorescence intensity (MFI) with whiskers for the standard error of the mean.

1b: Expression of CD11b, CD66 and CD62L on neutrophils sampled from donors or recovering HSCT patients and stimulated by germinating *Aspergillus* conidia. Long horizontal bars indicate mean fluorescence intensity (MFI) with short bars for the standard error of the mean.

1c: Surface expression of TLR2, TLR4 and Dectin-1 on neutrophils sampled from donors or recovering HSCT patients. Long horizontal bars indicate mean fluorescence intensity (MFI) with short bars for the standard error of the mean.

1d: ROS production by neutrophils according to different stimuli at different time points after HSCT. Bars represent mean fluorescence intensity (MFI) with whiskers for the standard error of the mean.

1e: ROS production by neutrophils sampled from donors or recovering HSCT patients and stimulated by germinating *Aspergillus* conidia. Long horizontal bars indicate the mean with short bars for the standard error of the mean.

*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

Figure 2: Ability of neutrophils to hamper *Aspergillus* hyphae growth is impaired during recovery from neutropenia. Isolated neutrophils sampled from donors, allogenic or autologous HSCT recipients during recovery were incubated with germinating conidia in a 96-well plate for 15 hours at 37°C with 5% CO_2. After washing, fungal growth was assessed by fluorescent probe. Growth inhibition was calculated as 1 – (ratio of the fluorescence intensity of the well with neutrophils and *Aspergillus* to that of the well with *Aspergillus* only) and expressed as a percentage. Figure 2a: Comparison of inhibition between neutrophils from 23

donors, 33 allogenic and 4 autologous graft recipients at a ratio of 16 neutrophils for one germinating conidia. Long horizontal bars indicate the mean with short bars for the standard error of the mean. Figure 2b: Paired comparison between neutrophils sampled from 19 HSCT recipients and their respective donors. **: $p<0.005$; ***:$p<0.001$

Figure 3: calcineurin inhibitors reduce the ability of neutrophils to inhibit *Aspergillus* hyphal growth. Figure 3a: Neutrophils sampled from patients with normal calcineurin inhibitor trough levels (i.e. >150 ng/mL for cyclosporine and >10 ng/mL for tacrolimus) are less capable of inhibiting *Aspergillus* growth than those retrieved from patients with low trough levels (i.e. <120 ng/mL for cyclosporine and <6 ng/mL for tacrolimus). Long horizontal bars indicate the mean with short bars for the standard error of the mean. Figure 3b: Correlation (Pearson test) between the percentage of inhibition of *Aspergillus* 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 or the day before neutrophil sampling, except for two patients for whom concentration values had been determined two days before sampling and one patient five days before.

Figure 4: A calcineurin inhibitor diminishes the ability of neutrophils to inhibit *Aspergillus* growth. Blood collected from healthy donors was incubated for two hours at 37°C with cyclosporine (CsA; final concentrations of 500 ng/mL and 1000 ng/mL) or equivalent DMSO vehicle as control. Subsequently isolated neutrophils were used for an *Aspergillus* growth inhibition assay. The neutrophils pre-incubated with cyclosporine showed decreased fungal

inhibition compared to the untreated controls. Long dashed bars indicate the mean with short bars for the standard error of the mean. *: $p<0.01$; **: $p<0.005$

Figure 5: Neutrophil death and NETs formation is decreased during recovery in allogenic HSCT recipients.

Neutrophils sampled from HSCT patients during recovery or from healthy donors were incubated with germinating *Aspergillus* conidia for 3 hours at 37°C with 5% CO_2 in a 96-well plate placed on a Zeiss Axio Microscope (Carl Zeiss, Germany). Pictures were taken every minute. Sytox green (Life Technologies) was added in each well at a final dilution of 1/5000 at the end of the experiment. Images were processed and hyphal lengths measured using ImageJ software. Quantification of NETs formation was evaluated as previously described by Papayannopoulos *et al* [9] with ImageJ.

5a. Growth of *Aspergillus* hyphae (in µm) after 3 hours of culture with or without neutrophils sampled from HSCT patients or controls. Length was measured for hyphae in contact with neutrophils and for those that were not. Inhibition of *Aspergillus* growth (in µm) by neutrophils requires contact and is altered in HSCT patients. Results are representative of experiments with 4 patients and 4 controls. Bars represent means with whiskers for the standard error of the mean.

5b. The death rate (assessed by Sytox green) in neutrophils sampled from controls is higher than in neutrophils collected from patients.

5c. Images of 2 independent experiments showing the typical aspect of NETosis in controls while the neutrophils of patients show aspects evocative of apoptosis/necrosis.

5d-e. NETs formation assessed by measurement of an area above 400 µm² is higher with control neutrophils than with HSCT patient neutrophils as assessed by the repartition of the Sytox-green-positive neutrophils according to the area of signal (5d) and the percentage of the

591 Sytox-green-positive neutrophils that underwent NETosis, i.e. with signal area >400 μm^2
592 (5e).
593
594 **Figure 6: evolution of *Aspergillus* hyphae growth inhibition by neutrophils sampled**
595 **from HSCT recipients.** Growth inhibition is impaired during recovery but restored after 10
596 months. Adjunction of corticoid in patients who developed graft versus host disease is related
597 with a trend toward a reduction of the ability of neutrophils to inhibit fungal growth. Bars
598 represent means with whiskers for the standard error of the mean. *: $p<0.01$; **: $p<0.005$; ns:
599 non-significant.

Figure 1a

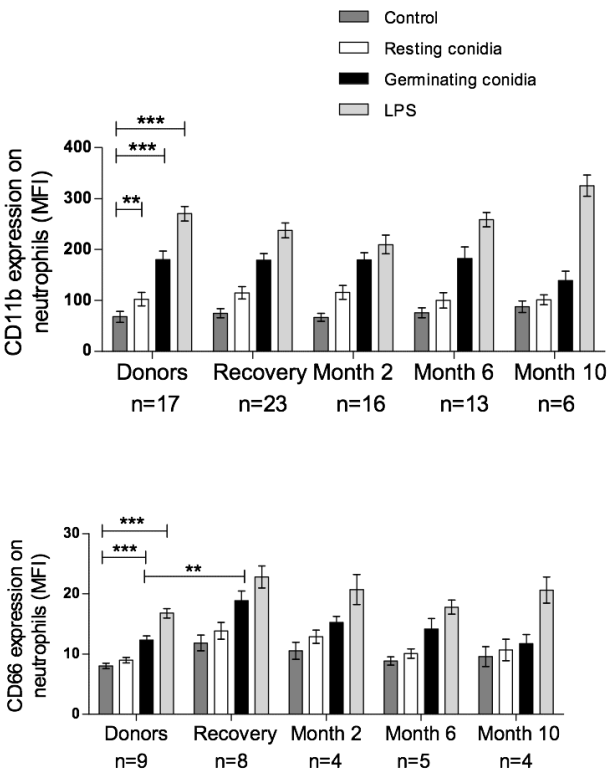


Figure 1b

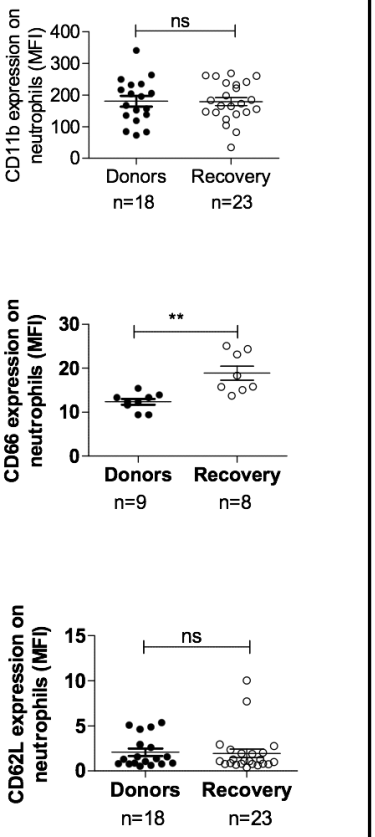


Figure 1c

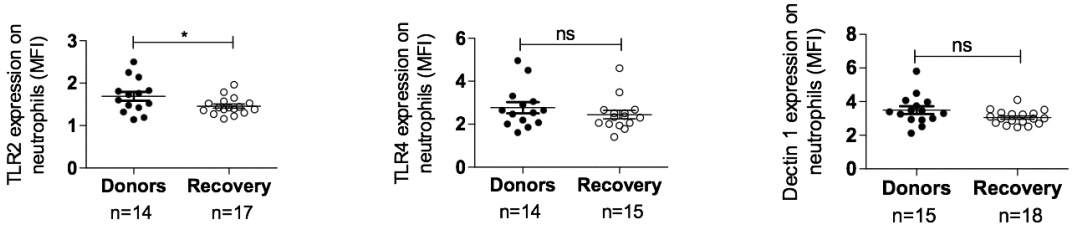
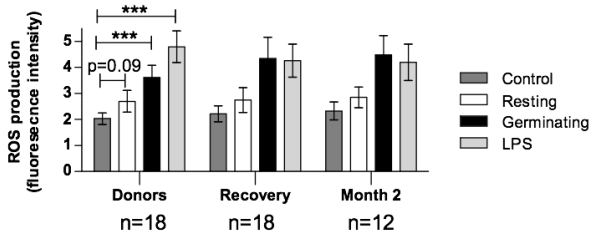


Figure 1d



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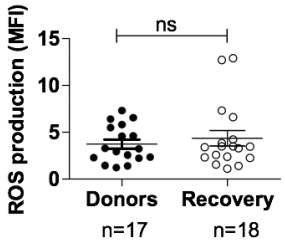


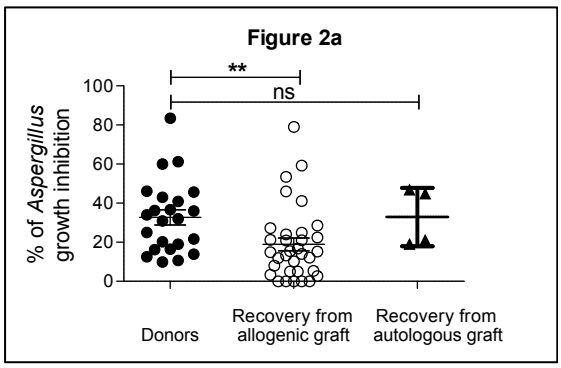


Figure 2a
**
ns
100
80
60
40
20
0
% of Aspergillus growth inhibition
Donors
Recovery from allogenic graft
Recovery from autologous graft

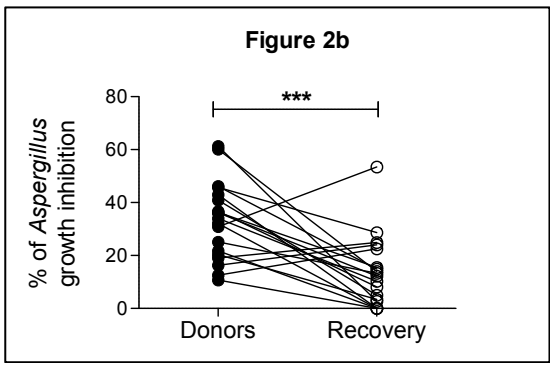


Figure 2b
***
80
60
40
20
0
% of Aspergillus growth inhibition
Donors
Recovery

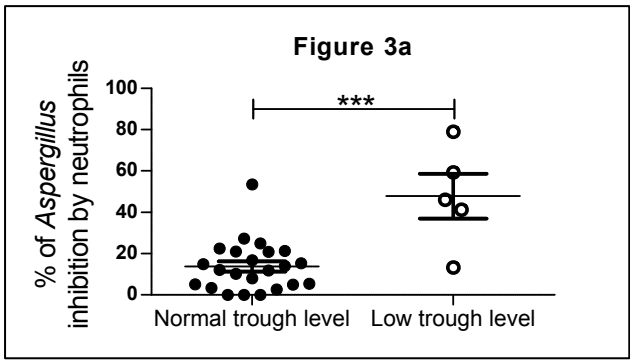


Figure 3a
% of Aspergillus inhibition by neutrophils
100
80
60
40
20
0
***
Normal trough level
Low trough level

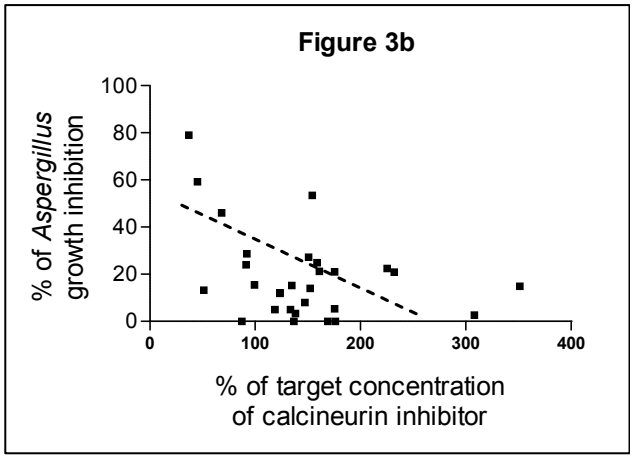


Figure 3b
% of Aspergillus growth inhibition
100
80
60
40
20
0
0
100
200
300
400
% of target concentration of calcineurin inhibitor

Figure 4

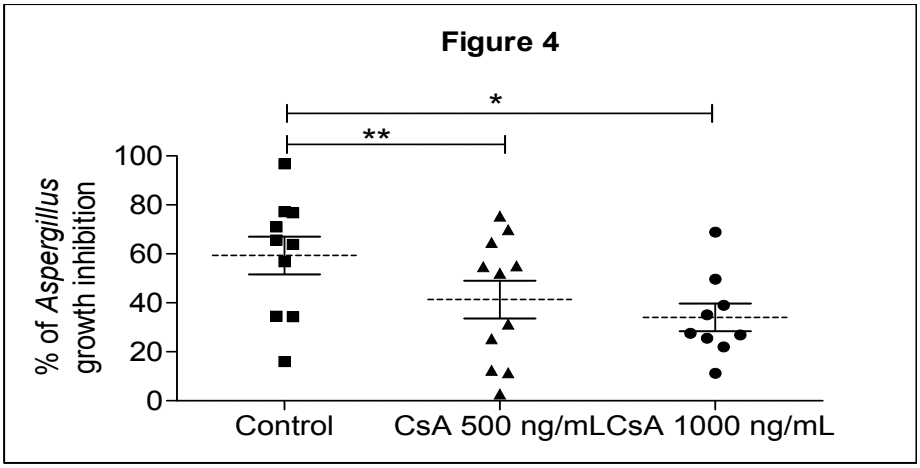

*
**
100
80
60
40
20
0
% of Aspergillus growth inhibition
Control
CsA 500 ng/mL
CsA 1000 ng/mL

Figure 5a

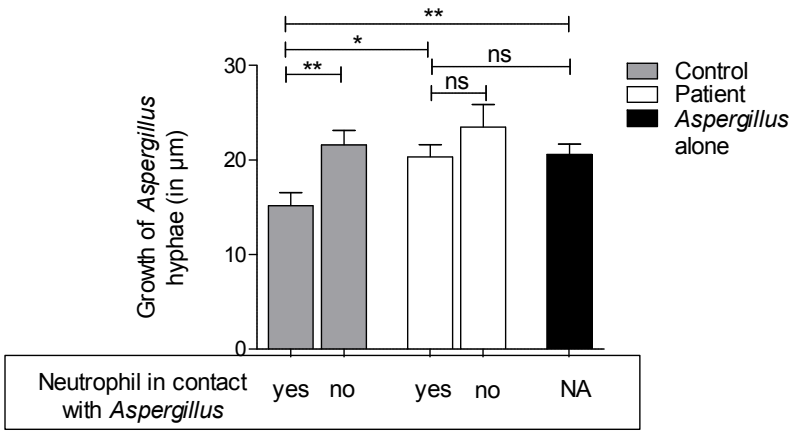


Figure 5b

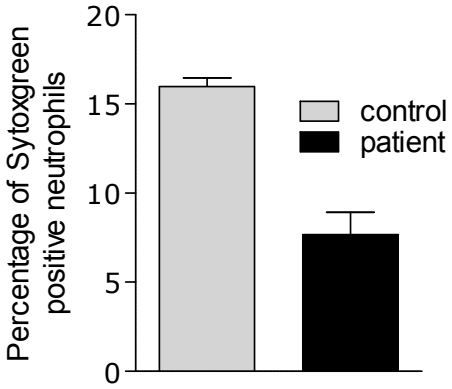


Figure 5c

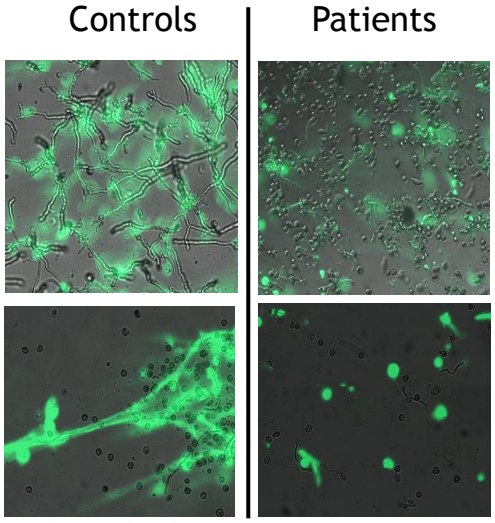


Figure 5d

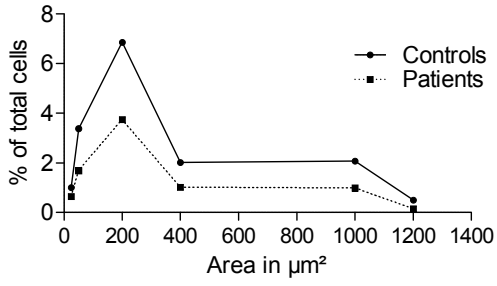


Figure 5e

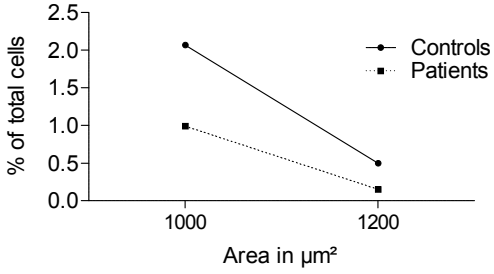
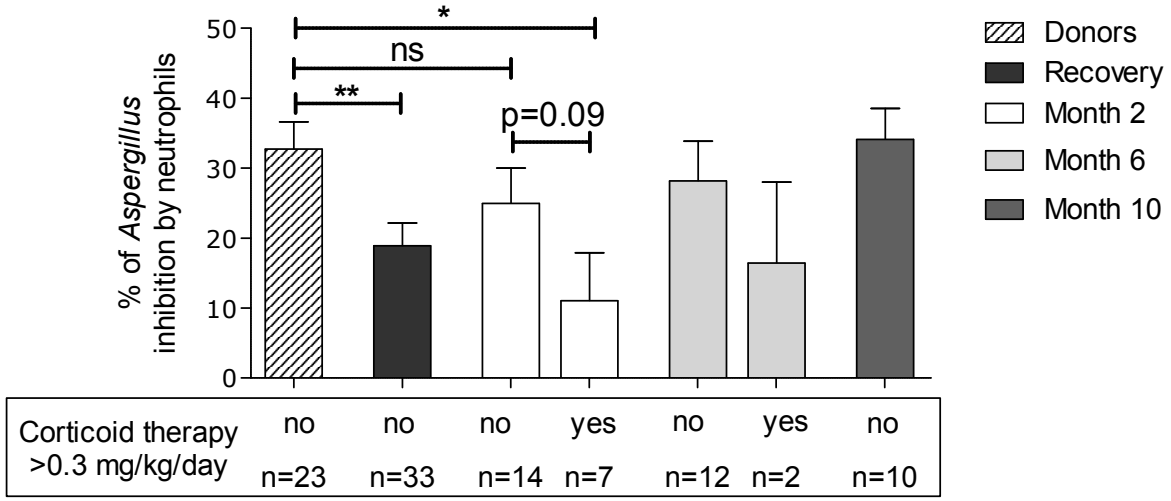


Figure 6


*
ns
**
p=0.09
50
40
30
20
10
0
% of Aspergillus inhibition by neutrophils
Donors
Recovery
Month 2
Month 6
Month 10
Corticoid therapy >0.3 mg/kg/day
no n=23
no n=33
no n=14
yes n=7
no n=12
yes n=2
no n=10