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

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2 genic hematopoietic stem cell transplant recipients**

3

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26 **ABSTRACT**

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

31 Objective: We aimed at assessing the activity of neutrophils on *A. fumigatus* in allogenic
32 HSCT recipients at different post-transplant time points.

33 Methods: We performed a longitudinal study involving 37 HSCT patients, drawing blood
34 samples at engraftment and at two, six and ten months after the HSCT. Post-transplant neu-
35 trophil activity in the recipients was compared to that of the respective donors. Neutrophil/
36 *Aspergillus* co-culture, flow cytometry and video microscopy were used to assess neutrophil
37 inhibition of fungal growth, cell/fungus interactions, reactive oxygen species production, ma-
38 jor surface molecule expression and neutrophil extracellular traps (NETs) formation.

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

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

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51 **KEY MESSAGES**

52 The ability of neutrophils to impair *Aspergillus* hyphal growth is altered during recovery in
53 allogenic hematopoietic stem cell transplant recipients.
54 The administration of calcineurin inhibitors seems to play an important role in this impair-
55 ment, in stark contrast to the classical view of these inhibitors as affecting only adaptive im-
56 munity.
57
58 **CAPSULE SUMMARY**

59 In allogenic hematopoietic stem cell transplant recipients, fungal innate immunity driven by
60 neutrophils is altered during the first months of the graft, in relation with the use of calcineu-
61 rin inhibitors.
62
63 **KEYWORDS**

64 Innate immunity; Calcineurin inhibitor; Invasive fungal infection; Immune reconstitution;
65 Transplantation
66
67 **ABBREVIATIONS**

68 HSCT: hematopoietic stem cell transplant
69 GvHD: graft versus host disease
70 NETs: neutrophil extracellular traps
71 ROS: reactive oxygen species
72
73 **INTRODUCTION**

74 *Aspergillus fumigatus* is the main causative agent of invasive aspergillosis, which is a major
75 threat to immunocompromised patients, including hematopoietic stem cell transplant (HSCT)

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76 recipients. Polymorphonuclear neutrophil cells (called neutrophils hereafter) are key effectors
77 against fungal infection. In contrast to monocytes and macrophages, which phagocyte resting
78 conidia, neutrophils are also able to act against germinating conidia and hyphae through a
79 trapping mechanism, giving them particular importance against *Aspergillus* [1, 2]. However,
80 only a few studies have provided data on the behavior of neutrophils following HSCT, and
81 additionally, how these cells regain their basic functions after the transplant is unknown.
82 The choice of immunosuppressors for the prevention of graft versus host disease (GvHD) can
83 vary depending on the conditioning regimen, the type of donor and the type of graft. The most
84 common prophylaxis strategy associates a calcineurin inhibitor (mainly cyclosporine A) with
85 methotrexate (MTX), anti-thymoglobulin antibodies (ATG) or mycophenolate mofetil (MMF)
86 [3]. Despite those immunosuppressive drugs, acute GvHD occurs in approximately 40-60% of
87 HSCT patients [3] and requires the administration of corticosteroids [3]. The action of cyclospor-
88 ine A, which is essentially known for its effect on adaptive immunity, is mediated by inhibi-
89 tion of calcineurin, a calcium-dependent phosphatase. Particularly, calcineurin activates the
90 NFAT transcription factors, leading to the transcription and production of many T-cell effec-
91 tor cytokines such as interleukin 2 [4-6]. Very few data exist concerning its potential role on
92 innate immunity. Patients receiving cyclosporine run an increased risk of viral, bacterial or
93 fungal infection. Among these pathogens, the mold *Aspergillus* is particularly dangerous,
94 causing high morbidity and mortality. The administration of cyclosporine or other recognized
95 T-cell immunosuppressors is included in the host criteria for probable invasive fungal disease
96 as established by the European Organisation for Research and Treatment of Cancer (EORTC)
97 and the Mycoses Study Group (MSG) [7].
98 Thus, for the present study, we aimed at evaluating neutrophil function and activity toward
99 *Aspergillus* in HSCT recipients over time. We assessed neutrophil activity, including fungal

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100 growth inhibition, oxidative burst, surface molecule expression, and neutrophil extracellular
101 traps (NETs) production, following different stimuli.
102
103 **MATERIALS AND METHODS.**
104 **Patients and donors.** The present longitudinal study involved 37 patients who received a
105 related (36/37) or unrelated (1/37) allogeneic HSCT for a malignant hemopathy in La Pitié-
106 Salpêtrière Hospital, Paris, France. Blood samples were collected from the recipients at en-
107 graftment, then at two, six and ten months post-transplant, and were compared to samples
108 taken from their respective donors. This non-interventional study was approved by the local
109 ethics committee (CPP Ile de France IV). Signed informed consent was obtained from all do-
110 nors and recipients.
111 **Neutrophil isolation.** Neutrophils were isolated using the dextran-Ficoll method. Briefly,
112 whole fresh blood was mixed with an equivalent volume of 2.0% dextran solution (Sigma
113 Aldricht) in normal saline and the red blood cells were allowed to settle for 40 minutes at
114 4°C. Then the leucocyte-rich supernatant was submitted to Ficoll (Eurobio) centrifuge separa-
115 tion for 30 minutes at 700 g; 4°C. After elimination of the remaining red blood cells, neutro-
116 phils in the pellet were recovered in RPMI medium and tested immediately.
117 ***Aspergillus* fumigatus strain.** An *A. fumigatus* sensu stricto strain isolated from clinical
118 samples in La Pitié-Salpêtrière Hospital was used. The strain was maintained on Sabouraud
119 with chloramphenicol and gentamicin agar tubes at 37° for 5-7 days. Conidia were harvested
120 with phosphate-buffered-saline (PBS) containing 0.05% Tween 20, washed three times and
121 suspended in PBS and counted.
122 ***Aspergillus* growth inhibition.** Black, 96-well clear-bottom plates (Greiner) were seeded
123 with 1,500 conidia per well in RPMI medium containing 1% fetal calf serum (FCS) and al-
124 lowed to germinate for 7 hours at 37°C. After this growth period, *Aspergillus* measuring ap-

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125 proximately 15-20 µm can be considered either as germinating conidia or as small hyphae.
126 The medium was then changed to RPMI without FCS and the isolated neutrophils were added
127 to the wells at different effector:target ratios in triplicate. In others experiments, neutrophils
128 were added directly to resting conidia. The plates were incubated overnight at 37°C with 5%
129 CO_2 then washed with purified water. Uvitex (1% w/v), a fluorescent marker of chitin (similar
130 to calcofluor, which has already been used to assess fungal biomass [8]) was added to the final
131 dilution. Finally, plates were read using a Flexstation analyzer with excitation at 350 nm and
132 emission at 435 nm. For a given effector:target ratio, the fungal growth was determined as the
133 ratio of the fluorescence intensity of the well containing neutrophils mixed with *Aspergillus* to
134 that of the well containing *Aspergillus* only. The percentage of inhibition was defined as 100
135 minus the percent of fungal growth.
136 **Surface molecule expression of neutrophils.** Five-hundred-microliter whole-blood samples
137 were stimulated with either 10^6 *Aspergillus* conidia (resting or germinating), 5 ng/mL bacteri-
138 al lipopolysaccharide (LPS) (Sigma Aldrich) or PBS as control for 45 minutes at 37°C. Neu-
139 trophils were stained with an anti-human CD11b (or integrin alpha M) antibody (Dako), an
140 anti-human CD62L (or L-selectin) antibody (Becton Dickinson) and/or an anti-human CD66
141 (or carcinoembryonic antigen) antibody (Becton Dickinson) before cytometry analysis. TLR
142 and dectin expression were assessed using anti-TLR2, anti-TLR4 and anti-dectin-1 antibodies
143 (RD Systems). Cytometry was performed on a Gallios flow cytometer and results were ana-
144 lyzed using Kaluza software (Beckman Coulter).
145 **Measurement of neutrophil oxidative burst.** Neutrophils contained in 500 µL heparinized
146 whole-blood samples were incubated with hydroethidine (Sigma Aldrich) (final concentration
147 1.5 µg/mL) for 15 minutes at 37°C, then stimulated with either 10^6 *Aspergillus* conidia (rest-
148 ing or germinating), 5 ng/mL LPS or PBS as control for 45 minutes at 37°C. Then PMA (final

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149 concentration: 10 µM) or PBS was added for 5 minutes. Samples were then analyzed by flow
150 cytometry.
151 **Video microscopy and NETs formation assessment.** Interactions between *Aspergillus* and
152 neutrophils were visualized using a Zeiss Axio Microscope (Carl Zeiss, Germany). After 3
153 hours of co-culture, Sytox green (Life Technologies) was added to each well at a final dilution
154 of 1/5000. Images were processed and hyphal length was measured using ImageJ software.
155 Quantification of NETs formation was evaluated as previously described [9] with ImageJ.
156 **Statistical analysis.** GraphPad Prism 5 was used for statistical analyses (GraphPad software,
157 La Jolla, Calif).
158
159 **RESULTS.**
160 **Patient characteristics.**
161 Patient data are presented in Table 1. Mean donor and recipient ages were 47 (range 19-67)
162 and 44 (20-69) years respectively. There were 23 male and 13 female donors and 19 male and
163 18 female recipients. No significant differences were observed concerning age (p=0.25 by
164 Student test) or sex ratio (p=0.39 by Chi-square test). The main single indication for HSCT
165 was acute myeloid leukemia (40.5 % of patients) but lymphoproliferative disorders (i.e. lym-
166 phoma, lymphoid leukemia and myeloma) collectively accounted for 43% of cases. Other
167 diseases included primary myelofibrosis and myelodysplastic syndrome.
168 The majority of patients (86.5%) were transplanted with a matched related donor. Condition-
169 ing regimens were mainly busulfan-based with reduced intensity (54.1%) or myeloablative
170 (29.7%). All patients received GvHD prophylaxis involving a calcineurin inhibitor-based reg-
171 imen plus other drugs depending on the type of graft and conditioning.
172 Neutrophils were collected from patients in the first month (recovery from neutropenia) and at
173 two, six and ten months post-HSCT. Recovery from neutropenia (“recovery” hereafter) was

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174 defined as the day where the neutrophil count became >500/mm3 (although for one patient
175 we found *a posteriori* that this value was not reached). At recovery (median 20 days), all pa-
176 tients were receiving a calcineurin inhibitor, usually cyclosporine (88.6%). No patients had
177 corticosteroid therapy. Due to patient death or loss to follow-up, 10-month samples were
178 available for only 10 patients. The characteristics of blood samples and immunosuppressive
179 drugs regimens at one, two, six and ten months post-HSCT are presented in Table 2. Only
180 donor-derived cells were detectable for all included recipients after approximatively two
181 months post-transplant (data not shown).

182

183 **Neutrophil surface molecule expression after HSCT**

184 The surface expression of CD11b (also known as integrin alpha M), CD62L (or L-selectin)
185 and CD66 was evaluated at the basal level and following stimulation by resting/germinating
186 conidia or LPS. In association with the β_2 integrin (or CD18), CD11b forms the heterodimeric
187 integrin macrophage-1 antigen involved in the adhesion and migration of leukocytes. CD11b
188 is expressed at the surface of neutrophils after degranulation as it is contained in secondary
189 and tertiary neutrophil granules. CD62L is involved in transient tethering of the neutrophils to
190 the endothelial surface. The shedding of CD62L marks an activation of the neutrophils. CD66
191 is specific to secondary granules [10]. Surface expression of the major pattern recognition recep-
192 tors TLR-2, TLR-4 and dectin-1 were also assessed by flow cytometry. Neutrophils were
193 found to be activated in only a limited manner by resting conidia but they were strongly acti-
194 vated by germinating conidia, as evidenced by an increase in CD11b and CD66 expression
195 and a decrease in CD62L expression (Figure 1a and data not shown). As tested, LPS induced
196 high activation. There were however no observed differences over time. It is also noteworthy
197 that no differences were observed between donors and patients during recovery except as
198 concerns the expression of CD66, which was higher after the graft (Figure 1 a-b). In compari-

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199 son with the donors, the expression of TLR4 and dectin did not change but TLR2 diminished
200 slightly in recipients during recovery (Figure 1c).
201
202 **Neutrophil reactive oxygen species production after HSCT.**
203 As it is a major anti-pathogen mechanism of neutrophils, the production of reactive oxygen
204 species (ROS) was analyzed at a basal level and following exposition to resting/germinating
205 conidia or LPS. Resting conidia caused a moderate production of ROS while germinating
206 conidia led to an important oxidative burst (Figure 1 d-e). The level of ROS production was
207 highly dependent on the stimulus ($p<0.0001$ by 2-way ANOVA), but it remained similar be-
208 tween recipients and their donors, and stable over time ($p=0.96$).
209
210 **Neutrophil inhibition of *Aspergillus* hyphae growth.**
211 Samples were available from 23 donors and 33 recipients at recovery to test *Aspergillus* hy-
212 phae growth inhibition by neutrophils. This test set also permitted 19 paired sample compari-
213 sons. Unpaired (figure 2a) and paired (figure 2b) analysis showed a highly statistically signif-
214 icant decrease of the ability of neutrophils to hamper fungal growth during the recovery,
215 compared with healthy donors.
216 Interestingly, there were no differences concerning the ability of neutrophils to inhibit the
217 development of resting conidia (data not shown). Of note, no correlations were observed be-
218 tween, on one hand, the period between engraftment and sampling, and on the other, the per-
219 centage of inhibition. There was also no link between the absolute neutrophil count at the
220 time of sampling and the ability of neutrophils to inhibit *Aspergillus* growth (data not shown).
221
222
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224 **Effect of calcineurin inhibitors on neutrophil impairment of *Aspergillus* hyphal growth.**

225 During the study, some patients in recovery had neutrophils that were as effective as controls
226 at inhibiting *Aspergillus* growth. This was not related to time to engraftment, type of graft,
227 type of conditioning, or absolute neutrophil count in blood. But interestingly, we found that
228 these patients had low plasma calcineurin inhibitor trough concentrations. Indeed, these
229 treatments are difficult to balance and therefore regularly monitored. Considering this, we
230 compared the neutrophils of patients with low plasma calcineurin inhibitor trough concentra-
231 tions (i.e. <120 ng/mL for cyclosporine and <6 ng/mL for tacrolimus) to those of patients with
232 normal trough concentrations (i.e. >150 ng/mL and >10 ng/mL) for *Aspergillus* inhibition and
233 found that this latter was significantly greater in patients with low plasma concentrations
234 (Figure 3a). Moreover, Pearson correlation analysis showed a certain link between the per-
235 centage of inhibition and the plasma calcineurin inhibitor trough level ($r = -0.39$; $p<0.05$)
236 (Figure 3b). To strengthen the hypothesis that the observed effect was due to a pharmacologi-
237 cal effect and not a generic effect of recovery, which is a very complex phenomenon, we per-
238 formed growth inhibition tests using neutrophils from patients recovering from autologous
239 stem cell transplantation, where calcineurin inhibitors are not administered. In this setting, we
240 found no defects in the ability of neutrophils to impair *Aspergillus* growth (Figure 2a).

241 We also performed *in vitro* experiments to further assess the hypothesis that calcineurin inhib-
242 itors were correlated with reduced neutrophil inhibition. When blood sampled from healthy
243 donors was incubated with cyclosporine at 37°C for 2 hours, the subsequently isolated neu-
244 trophils showed diminished activity in terms of *Aspergillus* growth inhibition, in comparison
245 to untreated controls (Figure 4). Cyclosporine thus appears to impair neutrophil activity
246 against *A. fumigatus* hyphae.

247

248 **Alterations to NETs production in early post-HSCT period.**

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249 Neutrophils can exert their function through different mechanisms including phagocytosis,
250 ROS production, degranulation and NETs production. As discussed above, neither ROS pro-
251 duction nor degranulation (investigated by CD11b and CD66 surface expression) were altered
252 in the present study. Neutrophils cannot phagocytize hyphae due to their size and support ad-
253 hesion but it has been shown that they produce NETs in contact with *Aspergillus* consequent-
254 ly inhibiting its growth [11, 12]. We thus investigated the production of NETs by neutrophils and
255 their effects on fungal growth in both HSCT patients and healthy donors.
256 Neutrophils were co-cultured with *Aspergillus* for three hours and NETs production was visu-
257 alized using Sytox green. In accordance with the results presented above, *Aspergillus* growth
258 as assessed by hyphal length measurement during the three-hour culture was greater in pa-
259 tients than in controls; inhibition of *Aspergillus* growth requires contact between the fungus
260 and neutrophils (Figure 5a). The DNA area of the Sytox-positive cells was measured as pre-
261 viously described [9] for NETs quantification. Controls had a greater number of Sytox-positive
262 cells than patients did (Figure 5b). The results thus indicated that neutrophils of patients pro-
263 duce less NETs than those of controls (Figure 5c-e).
264

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

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

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274 second, that these effects are not permanent, at least when treatment is stopped or decreased
275 within some number of months.
276
277 **DISCUSSION.**
278 The few studies that have focused on the behavior of neutrophils following HSCT provide a
279 range of results suggesting moderate to no alterations in oxidative burst or antimicrobial ac-
280 tivity [15]. For the present study, we aimed at evaluating neutrophil function in HSCT recipients
281 compared to that in healthy donors as concerns the major human pathogen mold *Aspergillus*.
282 We found that oxidative burst and surface molecule expression at basal levels and following
283 stimulation did not vary significantly over time, with the exceptions of CD66, which was
284 more highly expressed during recovery, and TLR2, which was slightly less expressed. How-
285 ever, our results show that the recovery period is associated with a dramatic decrease in the
286 ability of neutrophils to inhibit *Aspergillus* hyphae growth, and that the use of calcineurin
287 inhibitors may play a large role in this impairment. It is well known that HSCT recipients are
288 particularly at risk for invasive aspergillosis, not only during the neutropenia period but also
289 after engraftment. It should be acknowledged however that neutrophil recovery is not the
290 most at-risk period since antifungal prophylaxis is now widely used, and furthermore that the
291 use of a calcineurin inhibitor is probable not singly sufficient to trigger invasive aspergillosis
292 although cases of aspergillosis in patients receiving cyclosporine have been reported [16]. Nev-
293 ertheless, aspergillosis breakthrough in patients receiving antifungals is not rare [17, 18] and the
294 assessment of the ability of neutrophils to correctly impair (or not) *Aspergillus* growth could
295 be used to evaluate the risk of invasive aspergillosis in at-risk patients and thus contribute to
296 reducing the fungal risk. Nonetheless, calcineurin inhibitors do appear to add to other ac-
297 quired or potential innate immune deficiencies that, together, favor the appearance of fungal
298 disease, which can occur any time after HSCT. Indeed, approximately 20% of invasive mold

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299 infections are diagnosed early (<40 days after HSCT), 40% late (between 40 and 100 days
300 after HSCT) and 40% very late (>100 days after HSCT) [19]. Whether this defect favors the
301 occurrence of invasive aspergillosis and adds to genetic disorders (e.g. TLR-4 [20] or IL-1β [21])
302 will need to be assessed in further studies.

303 For years, calcineurin inhibitors have been thought to exert their activity almost exclusively
304 by targeting lymphocytes. However, over the past few years, a growing body of evidence
305 suggests that they also have important effects on innate immunity. In mouse models of inva-
306 sive aspergillosis, the administration of cyclosporine has been shown to shorten survival [22, 23].
307 However, non-concordant results have been reported in other animal studies and it remains
308 unclear if cyclosporine alone is sufficient to favor the development of invasive aspergillosis
309 [23, 24]. More recently, Greenblatt *et al* used *in vitro* and murine models to show that calcineurin
310 regulates neutrophil immunity against the yeast *Candida albicans* [5]. They reported that mice
311 treated with cyclosporine were more highly susceptible to disseminated *Candida* infection
312 than were controls and that both calcineurin deficient neutrophils and cyclosporine treated
313 neutrophils showed impaired response toward *Candida*. More recently, Tourneur *et al* showed
314 that cyclosporine impaired human neutrophil function, but their patients were kidney trans-
315 plant recipients who were also receiving corticoid therapy, a known modifier of neutrophil
316 function [25].

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

322 Calcineurin inhibitors modulate several pathways, including NFATc, NF-κB or AP-1 [28, 29].
323 Thus, they not only inhibit the phosphatase activity of calcineurin but also the peptidyl-prolyl

324 *cis-trans* isomerase activity of their respective receptor called immunophilin. It would be of
325 interest to test the range of inhibitors that target the complex calcineurin pathway[29] to uncover
326 the mechanism by which NETs are processed. It would also be of great interest to know
327 whether targeting one or the other of these pathways would inhibit only alloreactive
328 memory/effector T cells, therefore preventing rejection or GvHD, without impairing the in-
329 nate immunity necessary for infection prevention. For now, there is not enough data to accu-
330 rately describe the mechanism by which calcineurin inhibitors limit NETs formation. Addi-
331 tionally, published results often appear contradictory. Gupta *et al* found that NETosis was
332 reduced dramatically by cyclosporine and ascomycin (an analogue of tacrolimus,) while ra-
333 pamycin, which targets the mammalian target of rapamycin (mTOR), had only a small effect
334 [27]. Inversely, McInturff *et al* found that NETosis was inhibited by rapamycin but not by tacro-
335 limus [30]. We underline however that those two studies used different agents to induce NETo-
336 sis: interleukin-8 for the former and LPS for the latter. In our work, we found that neutrophils
337 sampled from patients in recovery were less efficient at inhibiting *Aspergillus* growth than
338 those collected from healthy donors. ROS production was not impaired during recovery and
339 no correlation was observed between the percentage of inhibition and the level of oxidative
340 burst following stimulation by germinating conidia (data not shown). In contrast, Stuehler *et*
341 *al* reported recently that the ROS production of neutrophils collected from HSCT patients and
342 stimulated by *Aspergillus* was altered 30 days after the graft [31]. Interestingly however, their
343 results also indicate that the percentage of fungal damage mediated by neutrophils was de-
344 creased in HSCT patients compared to controls, even in patients with normal ROS production
345 [31]. In accordance with that, it has been shown that cyclosporine does not impair ROS produc-
346 tion [23]. Finally, in our microscopy experiments, we observed that in co-cultures with *Aspergil-*
347 *lus*, neutrophils sampled from patients in recovery produced less NETs than those sampled
348 from healthy donors, with a higher rate of fungal growth. However, rather than a specific ef-

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349 fect on NETosis, it seems that this diminished NET production is more generally a reflection
350 of a lower neutrophil death rate. The implication of calcineurin in cell death was first reported
351 long ago[32] and the enhancement of cell survival with the use of calcineurin inhibitors has been
352 observed with different cellular types [33, 34].
353

354 **CONCLUSION.**

355 This study exposes a previously unknown deficiency in the antifungal response of innate im-
356 munity of grafted patients. In hematopoietic stem cell transplant recipients, neutrophil-driven
357 immunity against *Aspergillus fumigatus* is altered during the first month post-transplant; ad-
358 ministration of calcineurin inhibitors plays an important role in this impairment, in stark con-
359 trast to the classical view of these inhibitors as affecting only adaptive immunity. The specific
360 pathway by which these drugs alter neutrophil antifungal response and NETs formation must
361 now be investigated in further studies, with the particular goal of enabling the development of
362 more specific therapeutic alternatives capable of inhibiting alloreactive effector T cells with-
363 out impairing the basic functions of innate immunity cells. Attaining this goal will be chal-
364 lenging but it is also vital for improving care in both HSCT and solid organ transplants.
365

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368

369 **POTENTIAL CONFLICTS OF INTEREST.**

370 None for all authors.

371 **CONTRIBUTIONS.**

372 SI performed experiments, PB performed experiments, AB designed and performed micro-
373 scopic experiments and participated in writing the paper, LG performed experiments; LS, MU

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

378

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385

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491 Table 1: Characteristics of patients included in the study and their conditioning regimens
492 RIC: reduced-intensity conditioning; MAC: myeloablative conditioning

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

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

495

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.

506

		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 **Figure 1: Post-engraftment evolution of surface molecule expression and reactive oxy-**
516 **gen species production by neutrophils**.

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517 1a: Expression of CD11b and CD66 by flow cytometry. Neutrophils were collected at differ-
518 ent time-points after HSCT and stimulated with resting or germinating *Aspergillus* conidia, or
519 lipopolysaccharide (LPS). Bars represent mean fluorescence intensity (MFI) with whiskers
520 for the standard error of the mean.
521 1b: Expression of CD11b, CD66 and CD62L on neutrophils sampled from donors or recover-
522 ing HSCT patients and stimulated by germinating *Aspergillus* conidia. Long horizontal bars
523 indicate mean fluorescence intensity (MFI) with short bars for the standard error of the mean.
524 1c: Surface expression of TLR2, TLR4 and Dectin-1 on neutrophils sampled from donors or
525 recovering HSCT patients. Long horizontal bars indicate mean fluorescence intensity (MFI)
526 with short bars for the standard error of the mean.
527 1d: ROS production by neutrophils according to different stimuli at different time points after
528 HSCT. Bars represent mean fluorescence intensity (MFI) with whiskers for the standard error
529 of the mean.
530 1e: ROS production by neutrophils sampled from donors or recovering HSCT patients and
531 stimulated by germinating *Aspergillus* conidia. Long horizontal bars indicate the mean with
532 short bars for the standard error of the mean.
533 *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$
534

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

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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

<u>Figure 3</u>: 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.

<u>Figure 4</u>: 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

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566 inhibition compared to the untreated controls. Long dashed bars indicate the mean with short
567 bars for the standard error of the mean. *: $p<0.01$; **: $p<0.005$
568

569 **<u>Figure 5</u>: Neutrophil death and NETs formation is decreased during recovery in allo-**
570 **genic HSCT recipients.**

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

578 5a. Growth of *Aspergillus* hyphae (in µm) after 3 hours of culture with or without neutrophils
579 sampled from HSCT patients or controls. Length was measured for hyphae in contact with
580 neutrophils and for those that were not. Inhibition of *Aspergillus* growth (in µm) by neutro-
581 phils requires contact and is altered in HSCT patients. Results are representative of experi-
582 ments with 4 patients and 4 controls. Bars represent means with whiskers for the standard
583 error of the mean.

584 5b. The death rate (assessed by Sytox green) in neutrophils sampled from controls is higher
585 than in neutrophils collected from patients.

586 5c. Images of 2 independent experiments showing the typical aspect of NETosis in controls
587 while the neutrophils of patients show aspects evocative of apoptosis/necrosis.

588 5d-e. NETs formation assessed by measurement of an area above 400 µm² is higher with con-
589 trol neutrophils than with HSCT patient neutrophils as assessed by the repartition of the
590 Sytox-green-positive neutrophils according to the area of signal (5d) and the percentage of the

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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.

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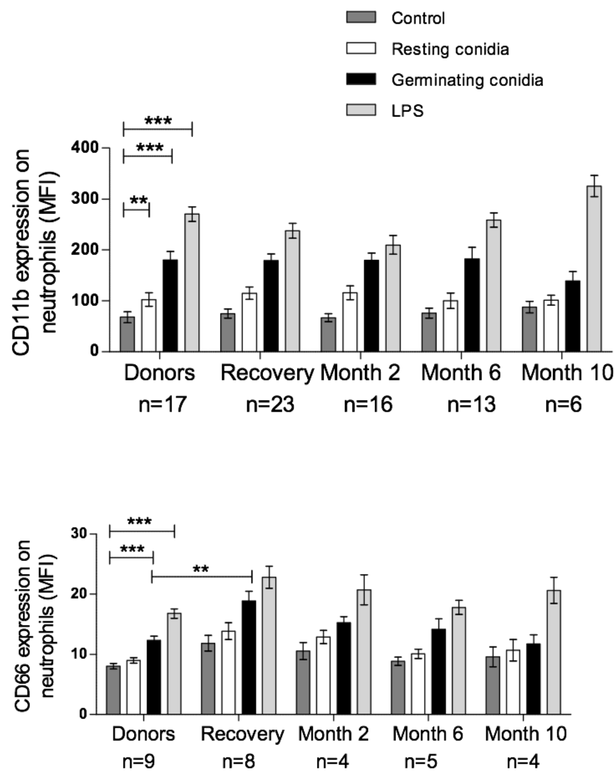
Figure 1a
Control
Resting conidia
Germinating conidia
LPS
CD11b expression on neutrophils (MFI)
Donors n=17
Recovery n=23
Month 2 n=16
Month 6 n=13
Month 10 n=6
CD66 expression on neutrophils (MFI)
Donors n=9
Recovery n=8
Month 2 n=4
Month 6 n=5
Month 10 n=4

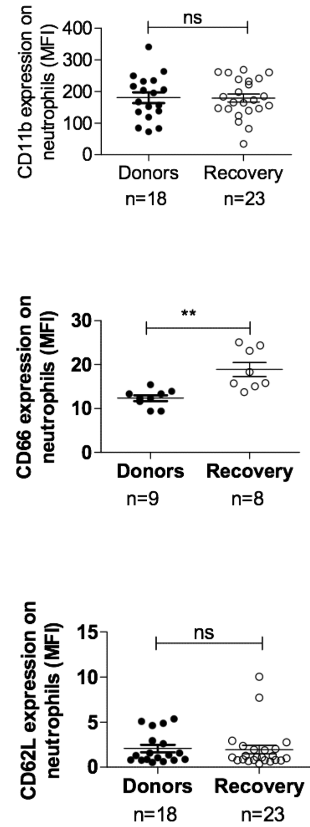
Figure 1b
CD11b expression on neutrophils (MFI)
ns
Donors n=18
Recovery n=23
CD66 expression on neutrophils (MFI)
Donors n=9
Recovery n=8
CD62L expression on neutrophils (MFI)
ns
Donors n=18
Recovery n=23

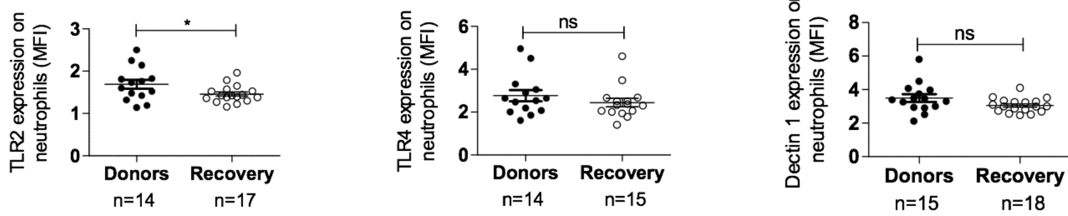
Figure 1c
TLR2 expression on neutrophils (MFI)
Donors n=14
Recovery n=17
TLR4 expression on neutrophils (MFI)
ns
Donors n=14
Recovery n=15
Dectin 1 expression on neutrophils (MFI)
ns
Donors n=15
Recovery n=18

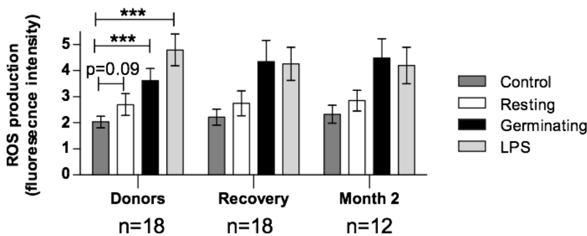
Figure 1d
ROS production (fluoresecnce intensity)
p=0.09
Control
Resting
Germinating
LPS
Donors n=18
Recovery n=18
Month 2 n=12

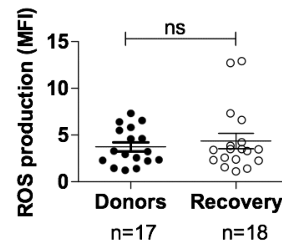
Figure 1e
ROS production (MFI)
ns
Donors n=17
Recovery n=18

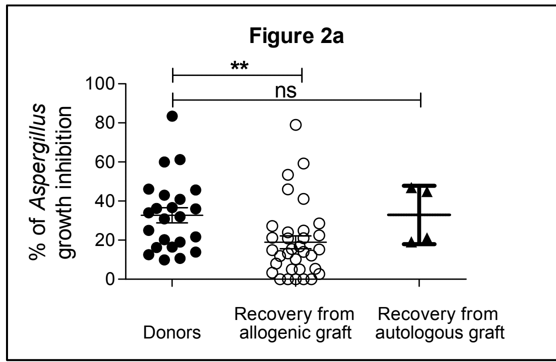
Figure 2a
**
ns
100
80
60
40
20
0
% of Aspergillus growth inhibition
Donors
Recovery from allogenic graft
Recovery from autologous graft

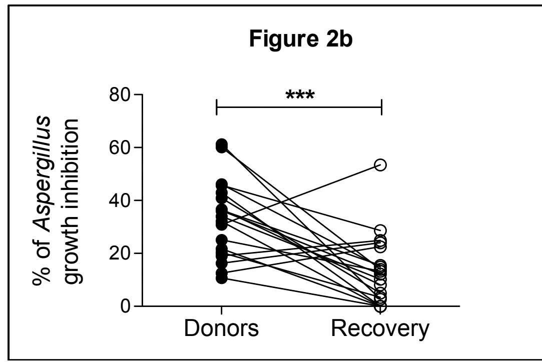
Figure 2b
***
80
60
40
20
0
% of Aspergillus growth inhibition
Donors
Recovery

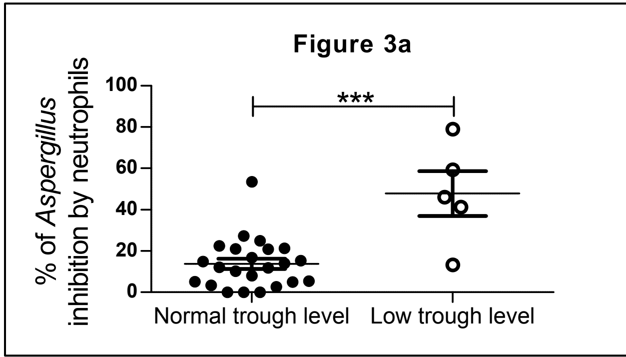
Figure 3a
% of Aspergillus inhibition by neutrophils
100
80
60
40
20
0
***
Normal trough level
Low trough level

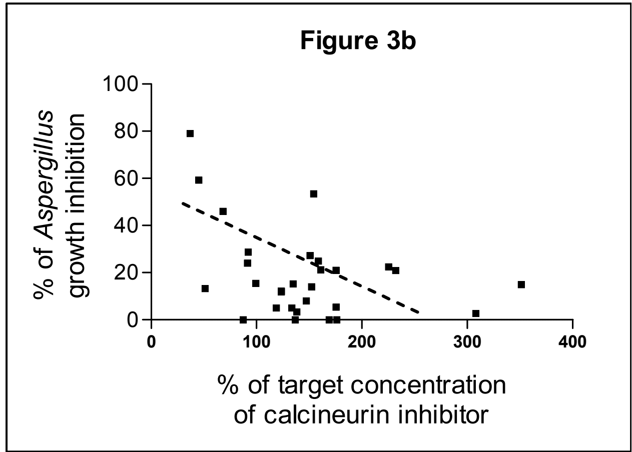
Figure 3b
% of Aspergillus growth inhibition
100
80
60
40
20
0
0
100
200
300
400
% of target concentration of calcineurin inhibitor

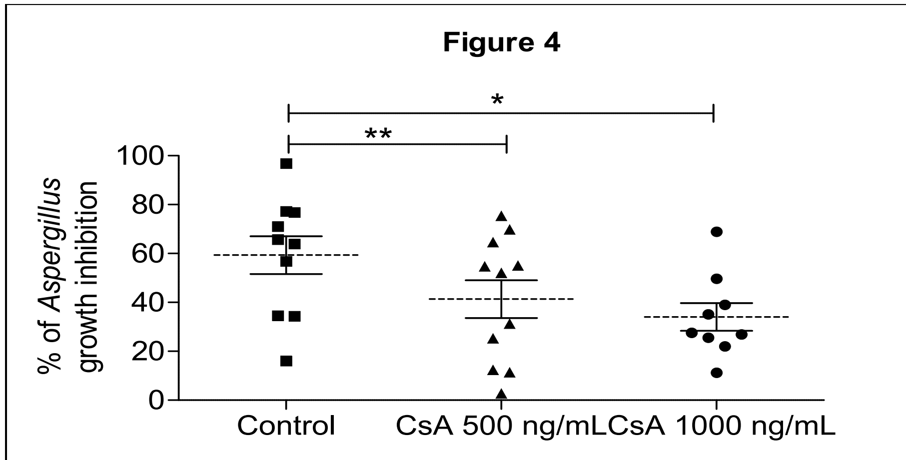
Figure 4
*
**
100
80
60
40
20
0
% of Aspergillus growth inhibition
Control
CsA 500 ng/mL
CsA 1000 ng/mL

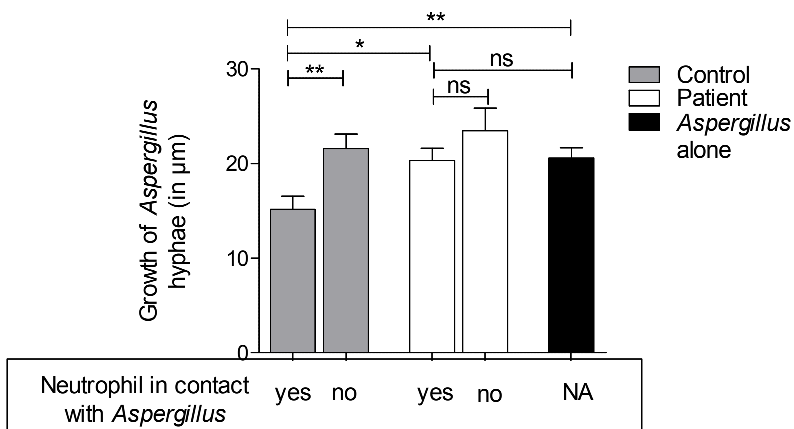
Figure 5a
Growth of Aspergillus hyphae (in µm)
**
*
ns
**
ns
30
20
10
0
Control
Patient
Aspergillus alone
Neutrophil in contact with Aspergillus
yes
no
yes
no
NA

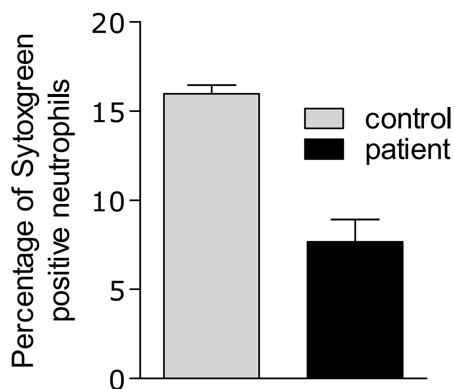
Figure 5b
Percentage of Sytoxgreen positive neutrophils
20
15
10
5
0
control
patient

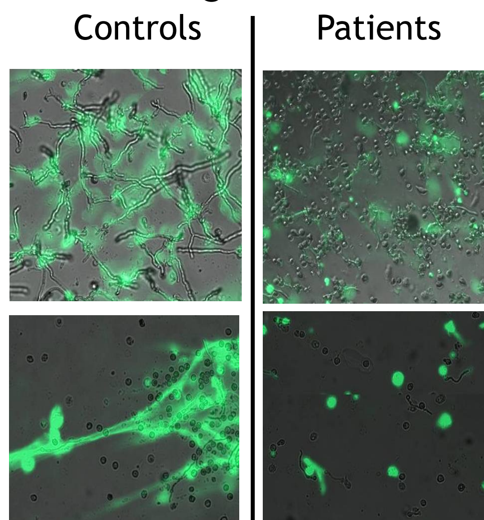
Figure 5c
Controls
Patients

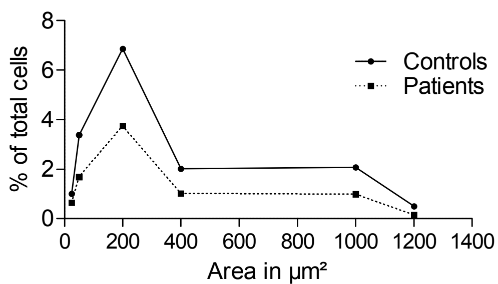
Figure 5d
% of total cells
8
6
4
2
0
Controls
Patients
0
200
400
600
800
1000
1200
1400
Area in µm²

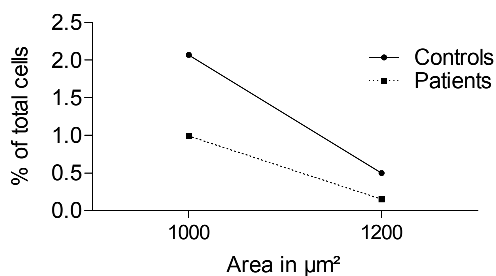
Figure 5e
% of total cells
2.5
2.0
1.5
1.0
0.5
0.0
Controls
Patients
1000
1200
Area in µm²

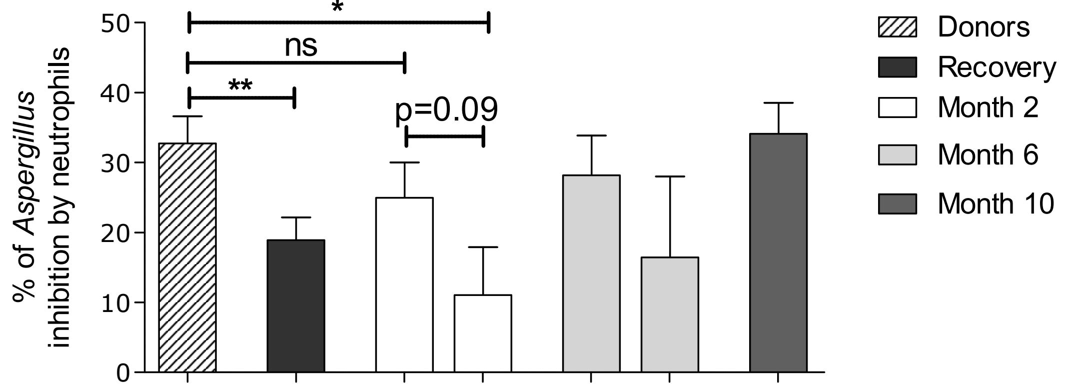
Figure 6
% of *Aspergillus* inhibition by neutrophils
50
40
30
20
10
0
*
ns
**
p=0.09
Donors
Recovery
Month 2
Month 6
Month 10
Corticoid therapy >0.3 mg/kg/day
no
no
no
yes
no
yes
no
n=23
n=33
n=14
n=7
n=12
n=2
n=10