

Immune Reconstitution After Allogeneic Hematopoietic Stem Cell Transplantation and Association With Occurrence and Outcome of Invasive Aspergillosis

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Background. Invasive aspergillosis (IA) remains a leading cause of morbidity and mortality in patients receiving allogeneic hematopoietic stem cell transplantation (HSCT). To date, no reliable immunological biomarkers for management and outcome of IA exist. Here, we investigated reconstitution of antifungal immunity in patients during the first 12 months after HSCT and correlated it with IA.

Methods. Fifty-one patients were included, 9 with probable/proven IA. We determined quantitative and qualitative reconstitution of polymorphonuclear (PMN), CD4, CD8, and natural killer (NK) cells against *Aspergillus fumigatus* over 5 time points and compared the values to healthy donors.

Results. Absolute CD4 and CD8 cell counts, antigen-specific T-cell responses, and killing capacity of PMN against *A. fumigatus* were significantly decreased in all patients over 12 months. In patients with probable/proven IA, reactive oxygen species (ROS) production tended to be lower compared to patients without IA, and absolute NK-cell counts remained below 200 cells/ μ L. Patients with well-controlled IA showed significantly higher ROS production and NK-cell counts compared to patients with poor outcome.

Conclusions. This study highlights the importance of functional PMN, T-cell, and NK-cell immunity for the outcome of IA. Larger multicenter studies should address the potential use of NK-cell counts for the management of antifungal therapy.

Keywords. antifungal innate and adaptive immunity; HSCT; immune reconstitution; NK-cell count as immunological marker.

Invasive aspergillosis (IA) remains a leading cause of morbidity and mortality in patients receiving allogeneic hematopoietic stem cell transplantation (HSCT) [1]. This infection occurs either early posttransplantation during neutropenia or late due to graft-versus-host

disease (GVHD) and the required immunosuppressive treatment [2].

Antifungal prophylaxis or treatment in high-risk patients is often ineffective due to impaired host immunity and is furthermore associated with drug interactions, emergence of resistant fungi, toxicity, and high costs [1, 3, 4]. Immune surrogate markers such as CD4⁺ cell counts in human immunodeficiency virus–infected individuals [5] and cytomegalovirus (CMV)–specific cell-mediated immunity in solid organ transplantation [6] to guide treatment have not been established for patients with IA.

Innate immune cells, including macrophages and polymorphonuclear cells (PMNs), are key players to control fungal invasion. PMNs possess an array of

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effector functions involving reactive oxygen species (ROS)-dependent and independent killing mechanisms [7, 8].

T cells, particularly CD4⁺ memory T cells, specific for different *Aspergillus* antigens can be detected in the peripheral blood of healthy individuals and HSCT recipients [9–11]. In general, T helper (T_H)1 and potentially T_H17 cytokines are considered to confer protective immunity against *Aspergillus*, whereas T_H2 responses are deleterious [12]. Furthermore, recent studies suggest that natural killer (NK) cells are crucial for fungal clearance [13–16].

So far, specific reconstitution of antifungal immune responses in patients after HSCT with and without IA has not been investigated in detail. The purpose of this study was to prospectively follow patients after HSCT and to obtain comprehensive data on immune reconstitution and the functionality of PMNs, NK cells, different T_H subsets, and CD8⁺ T cells in order to characterize susceptibility of these patients to IA and to identify possible biomarkers to guide antifungal treatment.

PATIENTS AND METHODS

Patients and Healthy Blood Donors

Adult patients undergoing allogeneic HSCT with peripheral blood stem cells at the University Hospital Basel from December 2012 to April 2013 were included. GVHD prophylaxis included cyclosporine A, methotrexate, and Cellcept in nonmyeloablative conditioning, and in some cases antithymoglobulin treatment. Antifungal prophylaxis consisted of fluconazole 400 mg once per week from conditioning to stop of immunosuppression. Pre-emptive and therapeutic antimold treatment consisted primarily of voriconazole 400 mg daily or higher doses according to therapeutic drug monitoring, posaconazole, or liposomal amphotericin B. GVHD treatment consisted of methylprednisolone 2 mg/kg/d. Corticosteroid refractory GVHDs were treated with alemtuzumab. All patient characteristics and follow-up information including occurrence of GVHD were retrospectively collected by chart review. Ethylenediaminetetraacetic acid blood was obtained before conditioning therapy and at day 30, 90, 180, and 360 after allo-HSCT. Additionally, 20 healthy donors were included as controls. The study was approved by the Ethic committee northwest and central Switzerland. Patients and healthy donors gave informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Definitions

Possible, probable, and proven IA cases were defined according to the consensus definitions developed by the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer and the Mycoses Study Group [17]. Definition of acute and chronic GVHD was according to the consensus workshop and the National Institutes of Health consensus criteria, respectively [18, 19].

Generation of Viable and Heat-Inactivated Fungi

Candida albicans ATCC60193 and *A. fumigatus* D141 were provided by Dr Reno Frei (University Hospital Basel, Switzerland) and PD Dr Sven Krappmann (Medical Immunology Campus Erlangen, Germany), respectively. Fungi were cultured as published [20] and heat-inactivated directly (*A. fumigatus* conidia) or after 15 hours' growth (*C. albicans* yeast and *A. fumigatus* hyphae).

Purification of PMNs and Peripheral Blood Mononuclear Cells

PMNs and peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood on a percoll gradient as published [21]. PMNs were incubated overnight in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) with 5% pooled human serum, and PBMCs were used directly after isolation.

Determination of ROS and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide Assay

ROS production of PMNs to *A. fumigatus* conidia (multiplicity of infection [MOI] 4) or *C. albicans* yeast (MOI 2) was determined using luminol-enhanced chemiluminescence as described [21].

Killing of *A. fumigatus* hyphae and *C. albicans* yeast by PMNs was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay based on published protocols [22]. Briefly, 3×10^4 outgrown *A. fumigatus* conidia or 3×10^5 *C. albicans* yeast were coincubated with PMNs for 2 hours at an effector:target ratio of 5:1 or 1:5, respectively. Amphotericin B (20 µg/mL, Sigma Aldrich) was used as positive control. After 2 hours, PMNs were lysed and an MTT solution (0.5 mg/mL in RPMI [Sigma Aldrich]) with 100 µM menadione (Applichem) added for 90 minutes. Formazan formation was quantified photometrically at 590 nm–650 nm. The hyphal damage was calculated as $(\text{optical density } \{\text{OD}\}_{\text{control}} - \text{OD}_{\text{test}}) / \text{OD}_{\text{control}} \times 100$.

Interferon- γ , Interleukin-17, and IL-4 Enzyme-Linked Immunospot and Flow Cytometry

Interferon- γ (IFN- γ), interleukin (IL)-17, and IL-4 enzyme-linked immunospot (ELISPOT) (Mabtech) were performed as described [20]. PBMCs were stimulated with *A. fumigatus* hyphae, *C. albicans* yeast (MOI 0.05), staphylococcal enterotoxin B (SEB; 0.5 µg/mL, Sigma Aldrich), CMV pp65 (0.05 µg/mL), adenovirus hexon protein (0.05 µg/mL) (both from JPT Peptide Technologies), or tetanus toxoid (7IE, Tetanol Pur, Novartis) for 72 hours. CMV pp65 was analyzed in CMV seropositive HSCT recipients, adenovirus hexon protein in CMV seronegative.

Lymphocyte subsets were determined flowcytometrically with anti-CD3-peridinin chlorophyll (PerCP), anti-CD4-PacificBlue, anti-CD8-allophycocyanin, and anti-CD56 fluorescein isothiocyanate (all Biolegend). Data were acquired on a BD LSRFortessa (BD Biosciences) and analyzed with FlowJo software vX.0.7.

Reference laboratory values validated at the Diagnostic Laboratory Hematology of the University Hospital Basel were used. The normal range for lymphocytes, CD4⁺ T cells, CD8⁺ T cells, and NK cells is 900–3300, 700–1100, 500–900, and 200–400 cells/ μ L, respectively.

For NK-cell proliferation, PBMCs were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE, Invitrogen) according to the manufacturer's instructions and stimulated with *A. fumigatus* hyphae for 8d. NK-cell proliferation was determined by staining with anti-CD3–PerCP and anti-CD56 phycoerythrin-Cy7 (Biolegend).

Statistical Analysis

Comparisons between 2 groups were performed with the 2-sided Mann–Whitney *U* test. $P \leq .05$ was considered statistically significant. Statistical analyses were done using GraphPad Prism 6.0f and Stata 13.1 software (StataCorp LP).

RESULTS

Patients' Characteristics

Of the 87 allogeneic HSCT performed between December 2012 and April 2013 at the University Hospital of Basel, 51 (59%) patients were included in the present study. All patients were enrolled before conditioning except for 1 patient with IA who was included post-HSCT. Proven and probable pulmonary IA were diagnosed in 3 and 6 HSCT recipients, respectively, and possible pulmonary IA in 16 patients (Table 1 and Figure 1). Of the 9 patients with probable or proven IA, 4 were diagnosed before HSCT during induction chemotherapy. These patients had persisting pulmonary lesions at HSCT and were under antifungal treatment after HSCT. One patient was diagnosed during neutropenia. Four patients were diagnosed during GVHD. IA was diagnosed 3 weeks, 10 weeks, and in 2 patients 6 months after HSCT, respectively. IA was diagnosed by growth of *A. fumigatus* in bronchoalveolar lavage in 4 patients, by galactomannan >1.0 in bronchoalveolar lavage in 2 patients, and by histology in 3 patients (Supplementary Table 1).

Neutrophil-Mediated Killing of *A. fumigatus* is Decreased for up to One Year After HSCT

First, we investigated the recovery of absolute PMN counts and their functionality after HSCT. Absolute PMN counts were comparable to healthy individuals 30 days after HSCT in 91.6% of patients, independent of fungal infection (Supplementary Figure 1).

Production of ROS in response to *C. albicans* yeast and *A. fumigatus* conidia was significantly decreased 30 days after HSCT compared to healthy donors ($P = .0008$ and $P = .052$, respectively). In patients without IA, ROS production normalized 90 days after HSCT but remained significantly reduced in patients with probable/proven IA over 6 months ($P = .04$) (Figure 2A).

As fungal killing is dependent on oxygen-dependent as well as oxygen-independent mechanisms, MTT assays to study PMN killing capacity were performed. PMN killing of *C. albicans* yeast recovered concomitantly with the improvement of ROS production, whereas killing of *A. fumigatus* hyphae remained significantly decreased up to 1 year after HSCT in 73% of patients, even in patients with recovery of ROS production ($P \leq .0086$ [d30–360], Figure 2B).

In conclusion, these data show that despite normalized PMN values, ROS production is critically impaired in patients with IA, and PMN-mediated killing of *A. fumigatus* remains significantly reduced over 1 year in all HSCT recipients.

CD8 and CD4 T-cell Recovery is Delayed in All HSCT Recipients and Patients With IA Show Impaired NK-cell Recovery

We next analyzed the recovery of the different lymphocyte subpopulations. Absolute CD8⁺ and CD4⁺ T-cell counts remained below the reference values over 12 months after HSCT independent of IA (Figure 3A and Supplementary Figure 1). NK-cell counts recovered to normal values 30 days after HSCT in patients without IA. In patients with probable/proven IA, NK-cell counts remained below the reference values for up to 180 days (median of 164 cells/ μ L compared to ≥ 200 cells/ μ L in healthy individuals; Figure 3A).

To determine if antigen-specific cytokine secretion by different T-cell subsets is affected after HSCT, we stimulated PBMCs with the mitogen SEB and different fungal (*A. fumigatus* hyphae, *C. albicans* yeast), bacterial (tetanus toxoid), and viral (CMV pp65, adenovirus hexon protein) antigens and determined IFN- γ , IL-17, and IL-4 secretion by ELISPOT. Mitogen-induced IFN- γ and IL-17 secretion was significantly decreased over 12 months after HSCT compared to healthy individuals (for IFN- γ , $P \leq .0003$ [d30–90]; for IL-17, $P \leq .0091$ [d30–360]), whereas IL-4 secretion showed almost normal levels. Compared to healthy donors, antigen-specific responses were significantly lower for all antigens and cytokines ($P \leq .015$) with the exception of some patients reactivating CMV that showed high IFN- γ responses toward CMV pp65 (Figure 3B and Supplementary Figure 2). The deficiency of antigen-specific responses was probably at least partly due to the quantitative T-cell deficiency. Moreover, the antigen-specific responses were not shifted toward IL-17 or IL-4 production.

These findings show that T-lymphocyte recovery and cytokine secretion is significantly impaired over 12 months after HSCT in all patients, rendering them susceptible to various infections. Low NK-cell counts may be associated with probable/proven IA.

Patients With Well-Controlled IA Show Higher NK-cell Counts and ROS Production Than Patients With Poor Outcome

To address the question if immunological markers differ in patients with probable/proven IA with favorable or poor outcome,

Table 1. Characteristics of Patients After Hematopoietic Stem Cell Transplantation

	Patients Without IA (n = 26)	Patients With Possible IA (n = 16)	Patients With Probable/Proven IA (n = 9)
Age (median, range)	45 (27–71)	46 (23–64)	55 (23–68)
Gender, male	16 (62%)	11 (69%)	6 (67%)
Underlying disease			
Acute myeloid leukemia	8 (31%)	7 (44%)	4 (44%)
Acute lymphoblastic leukemia	6 (23%)	2 (13%)	2 (22%)
MDS/MPS	5 (19%)	2 (13%)	0
Plasma cell disorders	1 (4%)	2 (13%)	1 (11%)
Chronic myeloid leukemia	4 (15%)	0	0
Lymphoma	0	1 (6%)	0
Chronic lymphocytic leukemia	0	1 (6%)	2 (22%)
Others	2 (8%)	1 (6%)	0
Donor source			
Matched unrelated donor	17 (65%)	11 (69%)	3 (33%)
Matched related donor	9 (35%)	5 (31%)	6 (67%)
Conditioning			
Myeloablative	19 (73%)	12 (75%)	6 (67%)
Nonmyeloablative	7 (27%)	4 (25%)	3 (33%)
Antithymocyte globulin	10 (38%)	10 (63%)	2 (22%)
GVHD ^a			
Acute GVHD Grade 1	6 (23%)	1 (6%)	3 (33%)
Acute GVHD Grade 2	6 (23%)	8 (50%)	0
Acute GVHD Grade ≥3	3 (11%)	3 (19%)	5 (56%)
Antimold therapy ^a			
Voriconazole	5 (19%)	15 (94%)	6 (67%)
Posaconazole	0	0	2 (22%)
Caspofungin	5 (19%)	1 (6%)	1 (11%)
Infectious complications ^a			
Other fungal infections ^b	1 (4%)	1 (6%)	3 (33%)
CMV replication	8 (31%)	8 (50%)	3 (33%)
Other viral infections ^c	0	0	1 (11%)
Bacterial infections ^d	1 (4%)	1 (6%)	2 (22%)
Immunsuppression ^a			
Cyclosporine A	26 (100%)	16 (100%)	9 (100%)
Corticosteroids	15 (58%)	10 (63%)	9 (100%)
Alemtuzumab	0	2 (13%)	2 (22%)
Mortality	7 (27%)	4 (25%)	5 (56%)

Abbreviations: CMV, cytomegalovirus; GVHD, graft-versus-host disease; IA, invasive aspergillosis; MDS/MPS, myelodysplastic and myeloproliferative syndrome.

^a Occurred at least once during study period.

^b *Candida parapsilosis*, *Candida albicans*, *Candida sake*.

^c Human herpesvirus 6, Epstein-Barr virus.

^d *Enterococcus faecium*, *Enterococcus faecalis*, *Klebsiella pneumoniae*.

we compared patients with recovery of IA (well-controlled IA) (n = 5) to patients with treatment failure and poor outcome (n = 4). Patients with well-controlled IA showed significantly higher ROS production to *A. fumigatus* ($P = .047$ [d90]), significantly higher lymphocyte counts ($P \leq .048$ [d90–360]), and significantly higher NK-cell counts ($P = .0082$ [d180]) compared to patients with poor outcome, suggesting that these cells may be essential for fungal control (Figure 4A and 4B

and Supplementary Figure 3). Interestingly, of 5 patients that developed IA after HSCT, 4 had NK-cell counts ≤ 66 cells/ μ L before disease development, suggesting that low NK-cell counts may predispose to IA. Additionally, in 3 of 4 patients, increasing NK-cell numbers coincided with the decrease of fungal lesions (Supplementary Table 1). Due to the low number of patients, it is unclear if patients with good outcome had a better T-cell response (Figure 4C and Supplementary Figure 3). However, 3 of

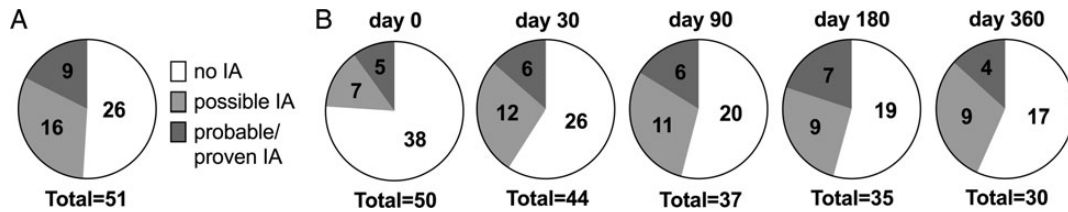


Figure 1. Patients included in the study. *A*, Total number of patients without invasive aspergillosis (no IA), possible IA, or probable/proven IA. *B*, Number of patients evaluated at the time of transplantation (day 0) and 30, 90, 180, and 360 days after HSCT. Patients were grouped at each time point according to current status of fungal infection. Twenty-one patients dropped out until day 360 because of death ($n = 16$) and loss of follow-up ($n = 5$). Abbreviation: HSCT, hematopoietic stem cell transplantation.

4 patients showed increased *A. fumigatus*-specific IFN- γ responses at the time of disease resolution (Supplementary Table 1).

Influence of Corticosteroid Treatment on Immune Reconstitution

It has been previously shown that corticosteroid treatment for GVHD management may impair PMN and T-cell function and delay NK-cell reconstitution [23–25]. We similarly saw that patients with GVHD and corticosteroid treatment tended to have diminished ROS production against *A. fumigatus* compared to patients without GVHD ($P = .1$ [d180]), whereas killing of *A. fumigatus* was decreased in all patients independent of corticosteroid treatment ($P \leq .022$ [d180]; Figure 5A). Corticosteroids had no significant influence on T-lymphocyte recovery and cytokine production over 1 year after HSCT compared to patients without GVHD. NK-cell counts, although not significantly,

were lower in patients with GVHD (Figure 5B and Supplementary Figure 4).

NK Cells of HSCT Recipients Have a Proliferation Defect in Response to *A. fumigatus*

As we have demonstrated that absolute NK-cell counts are decreased in patients with IA, we aimed to determine their functionality against *A. fumigatus*. For these experiments, we included 6 HSCT recipients without GVHD and 4 patients with GVHD. Compared to healthy individuals, NK-cell proliferation was significantly reduced in HSCT recipients with and without GVHD ($P = .015$ and $P = .039$, respectively; Figure 6) which was irrespective of absolute NK-cell counts. Patients with GVHD tended to have lower NK-cell proliferation than patients without GVHD ($P = .055$). Therefore, NK cells in HSCT recipients seem to have a proliferative defect in response to *A. fumigatus*.

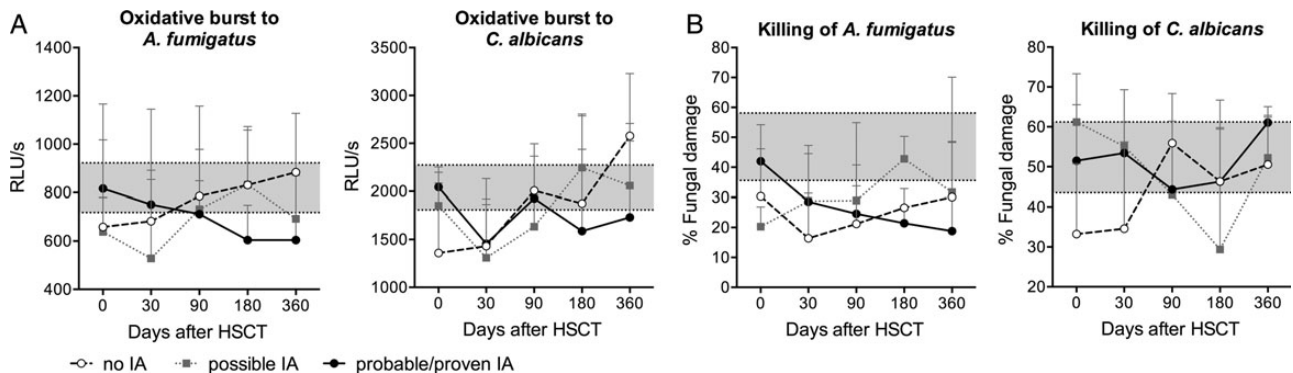


Figure 2. PMN-mediated killing of *A. fumigatus* is decreased for up to 1 year after HSCT. *A*, Maximum release of reactive oxygen species by PMNs after stimulation with heat-inactivated *A. fumigatus* conidia or *C. albicans* yeast in patients with no IA, possible IA, or probable/proven IA. Shown are median values + interquartile ranges of relative light units per second (RLU/s). The shaded areas indicate the 95% CI of medians of healthy controls ($n = 19$). No IA/possible IA/probable IA: $n = 24/6/4$ (d0), $n = 23/10/6$ (d30), $n = 20/10/6$ (d90), $n = 17/8/7$ (d180), $n = 16/8/4$ (d360). *B*, Percent fungal damage of *A. fumigatus* or *C. albicans* by PMNs in patients with no IA, possible IA, or probable/proven IA. *A. fumigatus* hyphae or *C. albicans* yeast were coincubated with PMNs for 2 hours and the percentage of viable fungi determined by MTT assay. The hyphal damage was calculated as $([OD_{\text{control}} - OD_{\text{test}}] / OD_{\text{control}}) \times 100$. Shown are median values + interquartile range. The shaded areas indicate the 95% CI of medians of healthy controls ($n = 15/18$ for *A. fumigatus*/*C. albicans*). No IA/possible IA/probable IA: for *A. fumigatus* $n = 12/5/3$ (d0), $n = 17/8/4$ (d30), $n = 11/6/4$ (d90), $n = 14/7/5$ (d180), $n = 15/7/3$ (d360); for *C. albicans* $n = 13/4/3$ (d0), $n = 15/9/2$ (d30), $n = 14/10/5$ (d90), $n = 15/7/5$ (d180), $n = 14/3/4$ (d360). Data are shown after subtraction of unstimulated controls. Abbreviations: CI, confidence interval; HSCT, hematopoietic stem cell transplantation; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; OD, optical density; PMN, polymorphonuclear cell.

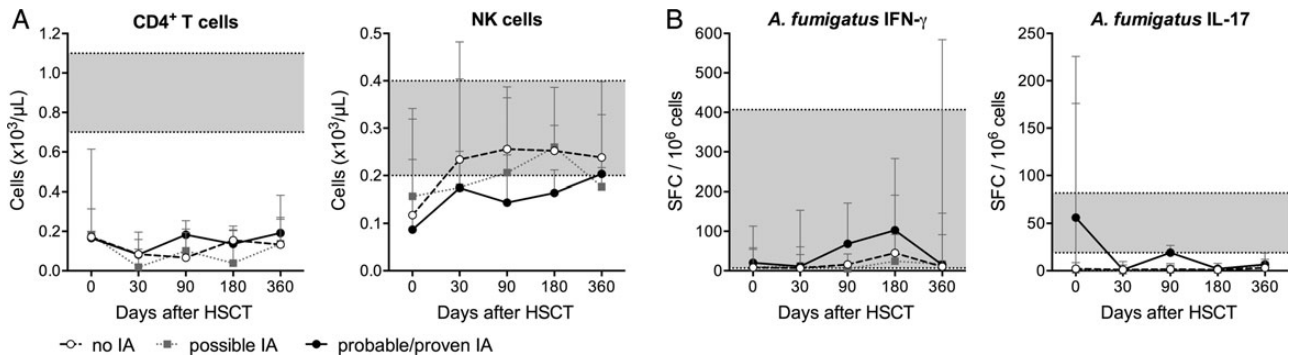


Figure 3. NK-cell counts are decreased in patients with probable/proven IA and *A. fumigatus*-specific cytokine secretion of PBMCs is reduced over 1 year after HSCT. *A*, Absolute CD4⁺ T cells and NK cells in patients with no IA, possible IA, or probable/proven IA. The absolute counts of CD4⁺ cells and NK cells were calculated from the percentage of CD3⁺CD4⁺ and CD3⁻CD56⁺ cells determined in flow cytometry, respectively, and the absolute lymphocyte counts. Shown are median values + interquartile ranges per μ L. The shaded areas indicate reference values used at the University Hospital Basel. No IA/possible IA/probable IA: n = 35/7/5 (d0), n = 24/11/6 (d30), n = 20/10/5 (d90), n = 19/9/7 (d180), n = 17/9/4 (d360). *B*, IFN- γ and IL-17 response to heat-inactivated *A. fumigatus* hyphae in patients with no IA, possible IA, or probable/proven IA. Cytokine response was determined by ELISPOT after 72 hours' stimulation. Shown are median values + interquartile ranges. The shaded areas indicate the 95% CI of medians of healthy controls (n = 20). No IA/possible IA/probable IA: for IFN- γ n = 31/6/4 (d0), n = 26/12/6 (d30), n = 20/10/4 (d90), n = 19/8/6 (d180), n = 16/9/4 (d360); for IL-17 n = 26/5/4 (d0), n = 25/9/6 (d30), n = 20/8/3 (d90), n = 18/6/6 (d180), n = 15/8/4 (d360). Data are shown after subtraction of unstimulated controls. Abbreviations: CI, confidence interval; ELISPOT, enzyme-linked immunospot; HSCT, hematopoietic stem cell transplantation; IA, invasive aspergillosis; IFN- γ , interferon- γ ; IL-17, interleukin-17; NK, natural killer; PBMCs, peripheral blood mononuclear cells; SFC, spot-forming cells.

DISCUSSION

In this study, we show that patients with probable/proven IA after HSCT have significant defects in PMN function to *A. fumigatus* and reduced restoration of NK-cell counts

over 1 year. These findings are further strengthened by the fact that in patients with well-controlled IA, these immunological parameters recovered faster than in patients with poor outcome. Moreover, NK-cell counts below 200 cells/ μ L were associated with probable/proven IA after HSCT.

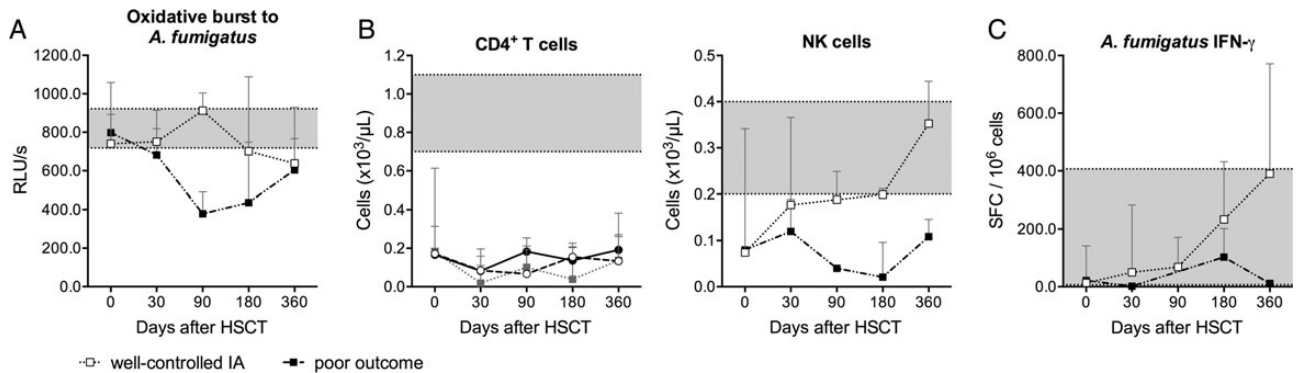


Figure 4. Patients with well-controlled IA show higher ROS production and NK-cell counts than patients with a poor outcome. *A*, Maximum release of ROS to *A. fumigatus* by PMNs in patients with probable/proven IA with well-controlled infection or poor outcome. Median values + interquartile ranges of relative light units per second (RLU/s) are shown. The shaded areas indicate the 95% CI of medians of healthy controls (n = 19). Well-controlled infection/poor outcome: n = 3/2 (d0), n = 4/3 (d30), n = 4/2 (d90), n = 3/3 (d180), n = 2/2 (d360). *B*, Absolute CD4⁺ T cells and NK cells in patients with probable/proven IA with well-controlled infection or poor outcome. The absolute counts of CD4⁺ cells and NK cells were calculated from the percentage of CD3⁺CD4⁺ and CD3⁻CD56⁺ cells determined in flow cytometry, respectively, and the absolute lymphocyte counts. Shown are median values + interquartile range per μ L. The shaded areas indicate reference values used at the University Hospital Basel. Well-controlled infection/poor outcome: n = 5/3 (d0), n = 4/3 (d30), n = 4/2 (d90), n = 3/3 (d180), n = 2/2 (d360). *C*, IFN- γ response to heat-inactivated *A. fumigatus* hyphae in patients with probable/proven IA with well-controlled infection or poor outcome. Shown are median values + interquartile ranges. The shaded areas indicate the 95% CI of medians of healthy controls (n = 20). Well-controlled infection/poor outcome: n = 4/3 (d30), n = 3/0 (d90), n = 3/2 (d180), n = 2/2 (d360). Data are shown after subtraction of unstimulated controls. Time points before development of probable/proven IA are also included. Abbreviations: CI, confidence interval; HSCT, hematopoietic stem cell transplantation; IA, invasive aspergillosis; IFN- γ , interferon- γ ; NK, natural killer; PMNs, polymorphonuclear cells; ROS, reactive oxygen species; SFC, spot-forming cells.

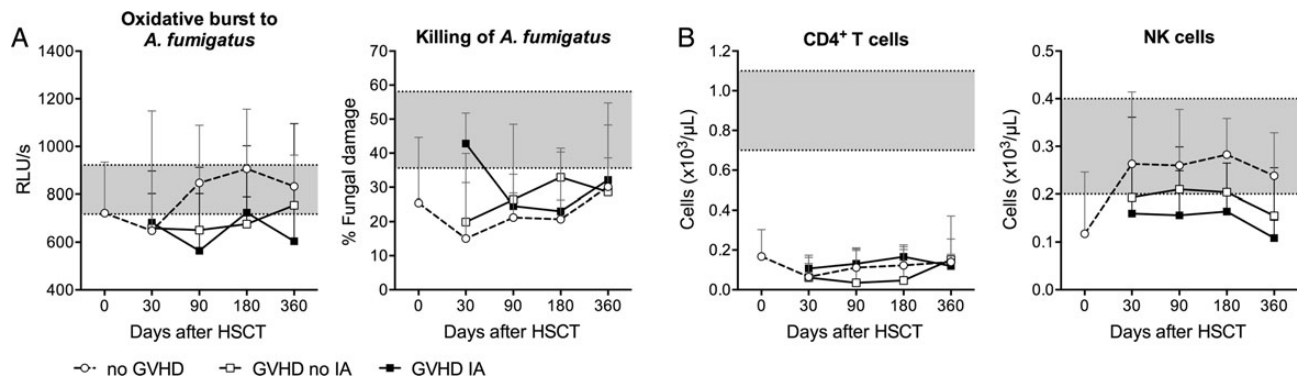


Figure 5. ROS production of PMNs to *A. fumigatus* is reduced and NK-cell recovery delayed in patients with GVHD receiving corticosteroid treatment. *A*, Maximum release of ROS to *A. fumigatus* and percent fungal damage of *A. fumigatus* by PMNs in patients without GVHD, with GVHD without IA, and with GVHD with IA. For ROS production, median values + interquartile ranges of relative light units per second (RLU/s) are shown. The shaded areas indicate the 95% CI of medians of healthy controls (n = 19). For fungal killing, percentage of viable *A. fumigatus* hyphae was determined by MTT assay. Shown are median values + interquartile ranges. The shaded areas indicate the 95% CI of medians of healthy controls (n = 15). No GVHD/GVHD no IA/GVHD IA: for ROS production n = 34/0/0 (d0), n = 22/14/5 (d30), n = 17/16/5 (d90), n = 20/10/4 (d180), n = 16/12/2 (d360); for killing n = 20/0/0 (d0), n = 18/10/2 (d30), n = 7/12/4 (d90), n = 13/10/4 (d180), n = 15/10/2 (d360). *B*, Absolute CD4⁺ T cells and NK cells in patients without GVHD, with GVHD without IA, and with GVHD with IA. The absolute counts of CD4⁺ cells and NK cells were calculated from the percentage of CD3⁺CD4⁺ and CD3⁻CD56⁺ cells determined in flow cytometry, respectively, and the absolute lymphocyte counts. Shown are median values + interquartile range per μL. The shaded areas indicate reference values used at the University Hospital Basel. No GVHD/GVHD no IA/GVHD: n = 46/0/0 (d0), n = 22/17/5 (d30), n = 17/15/4 (d90), n = 18/14/5 (d180), n = 17/12/2 (d360). Data are shown after subtraction of unstimulated controls. Abbreviations: CI, confidence interval; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; IA, invasive aspergillosis; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; NK, natural killer; PMNs, polymorphonuclear cells; ROS, reactive oxygen species.

Therefore, NK-cell counts may serve to guide antifungal treatment.

The strength of this study is the comprehensive longitudinal analysis of quantitative and qualitative innate and adaptive immunity specific for *A. fumigatus* in a large number of HSCT recipients. This allowed for identifying significant immunological

impairments in HSCT recipients compared to healthy individuals. To date, this is the largest prospective in-depth analysis of antifungal immunity of patients after HSCT. However, due to the low number of patients with IA, the differences between patients with and without IA rarely reached significance. Moreover, 8 of 9 patients with IA suffered concomitantly from GVHD and were under treatment with corticosteroids, making it difficult to distinguish between GVHD- or IA-related factors. As 5 of 9 patients developed IA before or early after HSCT, it is also uncertain whether the observed immunological defects predisposed to IA or if the fungal infection secondarily caused the immunological defects. Therefore, these data must be interpreted with caution and need further evaluation in larger studies.

We found that despite normal PMN counts, PMN function was significantly impaired after HSCT. Whereas PMN killing and ROS production to *C. albicans* recovered early after HSCT, PMN effector functions to *A. fumigatus* were impaired over 12 months, especially in patients with IA. This might explain why patients are more susceptible to mold infections for a prolonged period after HSCT [1, 26].

The CD4⁺ and CD8⁺ T-cell recovery and antigen-specific IFN-γ secretion to fungal, bacterial, and viral pathogens was significantly impaired over 12 months in all patients. Only some patients that reactivated CMV showed higher CD8⁺ T-cell counts and higher IFN-γ responses to the CMV pp65 peptide pool [27]. Similarly, we found that patients with IA showed

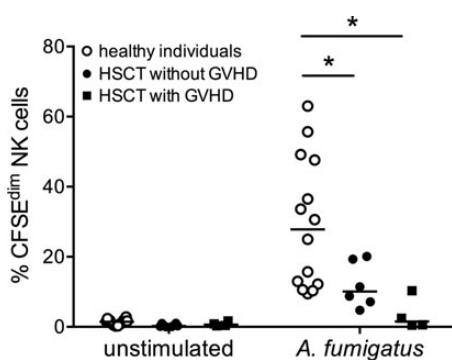


Figure 6. Patients after HSCT show decreased proliferation of NK cells to *A. fumigatus* hyphae. Proliferation of NK cells from healthy individuals (n = 14), HSCT recipients without GVHD (n = 6), and HSCT recipients with GVHD (n = 4) after 8 days' stimulation with *A. fumigatus* hyphae. The percentage of CFSE^{dim} cells in the CD3⁻CD56⁺ population was determined by flow cytometry (*P < .05). Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; NK, natural killer.

increased *A. fumigatus*-specific IFN- γ responses at the time of disease resolution. This indicates that an *A. fumigatus*-specific T_H1 response may be beneficial for fungal clearance. This correlation of a T_H1 immune response with better clinical outcome has been previously reported [20, 28–30] and encourages the development of antifungal T-cell transfer.

Previous studies reported that T_H17 responses are beneficial for fungal control, although data for *A. fumigatus* are controversial [12, 31], and that a high T_H2-to-T_H1 ratio is detrimental for fungal infection [12, 28]. In our study, all cytokines after antigen-specific stimulation were low, indicating that the risk for infection is probably due to an overall lack of specific immune responses and delayed T-cell recovery and not because of a disbalance in the cytokine milieu. Nevertheless, this should be interpreted with caution, as we did not investigate T-cell immunity at the site of infection (ie, lung).

We further identified an association of decreased NK-cell counts with probable/proven IA. Interestingly, most of the patients that developed IA after HSCT had very low NK-cell counts before disease development, and increasing NK-cell numbers coincided with the decrease of fungal lesions, indicating that low NK-cell counts may correlate with outcome and possibly also the development of IA. The role and mechanism of NK cells against *A. fumigatus* in human fungal infections has been scarcely investigated. Previous mouse studies reported that NK-cell recruitment is essential for antifungal defense in neutropenic animals [15] and that NK-cell proliferation is associated with inhibition of fungal growth [32]. Moreover, adoptive NK-cell transfer led to enhanced fungal clearance in neutropenic mice [13]. In healthy individuals, NK cells exert direct and indirect antifungal activity [14, 33]. As patients with isolated NK-cell deficiencies have no increased susceptibility for fungal infections [16, 34], NK cells probably need to interact with other immune cells for fungal control. Additionally, many fungal pathogens have immunomodulatory properties with suppressive effects on phagocytes, T cells, and NK cells [35, 36]. Therefore, the observed NK-cell impairments could be a consequence as well as a cause of IA.

Consistent with previous studies, we confirmed that patients receiving corticosteroids for the management of GVHD tended to have decreased effector functions of PMNs against *A. fumigatus*, particularly with respect to ROS production, confirming that these patients are highly susceptible to IA [2, 37]. Previous studies also reported an association of GVHD with low NK-cell counts [25]. In this study, we found only a slight reduction of NK-cell counts in patients with GVHD but a significant decrease of NK-cell proliferation after stimulation with *A. fumigatus*, which may be associated with higher susceptibility to IA.

Biomarkers to predict IA and to guide antimold prophylaxis and treatment would be desirable. Encouraging progress has been made in the identification of genetic factors implicated in the development of IA [38]. However, immunological factors

have so far not been investigated. In this study, we found that (1) ROS production was significantly lower in patients with IA, (2) NK-cell counts of patients with IA remained below 200 cells/ μ L for up to 6 months after HSCT, and (3) increasing NK-cell counts coincided with the decrease of fungal lesions and were associated with a good outcome. Due to the convenience of measuring and validating NK-cell counts, they would be ideal as immune biomarker to initiate antimold prophylaxis and guide antifungal treatment.

In conclusion, in this comprehensive analysis of antifungal immune responses after HSCT the importance of functional PMN, T-cell, and NK-cell immunity is shown. We demonstrate that the absolute NK-cell counts may be valuable as biomarker for the management of IA therapy. However, larger multicenter studies are needed to validate its use in the clinics.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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