ANALYSIS OF T-CELL RESPONSES TO ASPERGILLUS FUMIGATUS ANTIGENS IN HEALTHY INDIVIDUALS AND PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

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Abstract

Invasive Aspergillosis has become a major cause of infection-related mortality in non-neutropenic patients after allogeneic stem cell transplantation (SCT). To assess the potential role of Aspergillus-specific T-cell responses for the successful control of invasive aspergillosis, lymphoproliferative responses to A. fumigatus antigens were studied in healthy individuals, patients with evidence of invasive aspergillosis, and patients late after allogeneic SCT.

In healthy individuals, a positive lymphoproliferative response was documented to cellular extracts of A. fumigatus (14/16), the 88 kDa dipeptidylpeptidase (4/16), and the 90 kDa catalase (8/11). A predominant release of IFN-γ in culture supernatants upon stimulation with A. fumigatus antigens was demonstrated in 13/17 healthy individuals, indicating a T H 1 response. In patients with clinical evidence of invasive aspergillosis, a favourable response to antifungal therapy was found to correlate with a higher IFN-γ/IL-10 ratio in culture supernatants (n=7, median ratio IFN-γ/IL-10=1.0; range 0.09–24.8), compared to 10 patients with progressive or stable disease (median ratio IFN-γ/IL-10=0.1; range 0.002 –2.1) (P=0.04).

Steroid treatment was found to suppress A. fumigatus-specific lymphoproliferation (P=0.037) and release of IFN-γ in culture supernatants (P=0.017). In contrast to CMV- and tetanus toxoid-specific T-cell responses, Aspergillus-specific T-cell reconstitution late after allogeneic SCT was characterized by low stimulation indices and a low IFN-γ/IL-10 ratio. In addition, phosphoantigen-reactive Vγ9/Vδ2 T-cell clones from healthy individuals were found to produce significant amounts of TNF in response to Aspergillus fumigatus antigens.

In conclusion, these results further support the hypothesis that T cells contribute to the host defense against Aspergillus fumigatus.
Introduction

Invasive aspergillosis has become a major cause of infection-related mortality in patients with hematological malignancies, especially after allogeneic stem cell transplantation (SCT) (1). In the immunocompromised patient, invasive aspergillosis most frequently affects the lungs characterized by hyphal invasion and destruction of pulmonary tissue. Proven risk factors in humans are defects in phagocyte function (2), steroid-induced suppression of macrophage conidiocidal activity (3), and chemotherapy-induced neutropenia (4). More recently, invasive aspergillosis has been reported with an increasing frequency in non-neutropenic patients with advanced AIDS (5, 6), in preterm neonates (7), and in patients after solid organ transplantation and allogeneic SCT (1).

Airborne transmission of fungal spores has been considered to be the major route of transmission of invasive aspergillosis in the immunosuppressed host. Patients with a previous history of invasive aspergillosis have been found to be at an increased risk for recurrence of invasive aspergillosis during a subsequent episode of neutropenia or immunosuppression (8). These data and results from our group demonstrating Aspergillus-DNA in lower respiratory tract samples to be an important risk factor for the development of invasive aspergillosis during a subsequent episode of immunosuppression indicate that a subset of patients is obviously colonized without signs of tissue-invasive disease (9). These observations implicate, that local cellular defects in the innate and adaptive immune effector mechanisms are major predisposing factors of the host to invasive aspergillosis (3, 10, 11).

In the murine model of invasive pulmonary aspergillosis, resistance to the infection was associated with TNF-α, IL-12 and IFN-γ production (10, 12, 13). Production of the Th2 cytokines IL-4 and IL-10 by interstitial lymphocytes was associated with disease progression (10, 12). The development of Th1 protective immunity also correlated with resistance to subsequent lethal infection (12, 14). More recently, the adoptive transfer of CD4+ splenocytes from mice sensitized to a crude culture filtrate of A. fumigatus into naïve animals was found
to significantly prolong survival after a subsequent intravenous challenge with *A. fumigatus* conidia (15).

In the study reported, we assessed the lymphoproliferative T cell response to various *A. fumigatus* antigens in healthy individuals, patients with evidence of invasive aspergillosis, and patients late after allogeneic SCT. In the vast majority of healthy individuals and in patients surviving invasive aspergillosis, a significant lymphoproliferative response to *A. fumigatus* proteins was found with a dominant release of IFN-γ in culture supernatants. In patients late after allogeneic SCT, *Aspergillus*-specific T-cell reconstitution was characterized by low stimulation indices and a low IFN-γ/IL-10 ratio. Thus, this is the first study reporting on the important role of a T H 1 type cellular immune response for the control of invasive aspergillosis in patients with hematological malignancies. In addition to T helper cell responses, also phosphoantigen-reactive Vγ9/Vδ2 T-cell clones were found to produce significant amounts of TNF in response to *Aspergillus fumigatus* antigens.

**Material and Methods**

**Cell preparation**

Peripheral blood mononuclear cells (PBMNC) of healthy donors and hematological patients with clinical evidence of invasive aspergillosis were separated by use of ficoll-hypaque density gradient centrifugation (LINARIS, Bettingen am Main, Germany), washed twice in sterile calcium- and magnesium-free Hanks’ balanced salt solution (PAA Laboratories GmbH, Austria), and resuspended in RPMI 1640 medium supplemented with Glutamax-I, 25 MM HEPES buffer (GIBCO BRL), 200 µg/ml Gentamicinsulfate (Refobacin® 80 mg, Merck, Darmstadt, Germany), and 10% fetal calf serum (FCS, Sigma, St. Louis). All cell preparations were >95% viable as judged by trypan blue dye.

*Aspergillus fumigatus* antigens
Conidia of *A. fumigatus* (Strain CBS 144-89) were inoculated in 150-ml Erlenmeyer flasks containing Sabouraud liquid medium (2% [wt/vol] glucose, 1% [wt/vol] mycopeptone). Flasks were shaken for 24 h at 37°C and 200 rpm. Two-liter fermenters (LSL Biolafitte, Saint Germain en Laye, France) containing 1.2 liters of Sabouraud medium were inoculated with the shaken flask cultures. The 18 h-culture conditions were as follows: inoculum 8% (v/v); temperature, 26°C; aeration, 50 l of air per minute; agitation, 500 rpm. The mycelial mat recovered by filtration was extensively washed with water. Mycelium was disrupted in a glass bead cell homogenizer in 50 mM Tris HCl buffer pH 7.5 and the water-soluble cellular extracts (EC SAB) were recovered after centrifugation. Protein content was measured by the BioRad technique according to the manufacturer’s instructions and estimated in mg equivalent BSA/ml.

Conidia of *A. fumigatus* were harvested after 3 days of culture on Sabouraud dextrose agar (Difco, Detroit), filtered through sterile gauze, killed by heating in a water bath at 100°C for 1 h, washed with saline solution and stored at –4°C. Heat-killed *A. fumigatus* conidia were tested for sterility by subculturing on Sabouraud dextrose agar for 10 days.

The two major antigens of *A. fumigatus*, the monomeric 88 kDa dipeptidylpeptidase V (DPP V) and the 360 kDa catalase, a tetrameric protein with 90 kDa subunits, were expressed as recombinant proteins in *Pichia pastoris* and used for T cell stimulation (16, 17).

**Lymphoproliferation assay**

The proliferation assay was performed as described before (18). *A. fumigatus* antigens were added to the wells in concentrations ranging from 50 µg to 50 ng protein/ml. Conidia were tested in lymphoproliferative assays at concentrations ranging from $5 \times 10^5$ to $5 \times 10^2$. Tetanus toxoid (Chiron Behring GmbH, Marburg, Germany), CMV antigen (Biodesign, Dunn, Asbach, Germany), PHA (Murex, Life Technology, Karlsruhe, Germany), and IL-2 (Biotest, Dreieich, Germany) were used as control T cell stimuli and added at final concentrations of...
20 µg/ml, 10 ng/ml, and 50 U/ml respectively. T cells were stimulated with antigen for 5 days and 37 KBq \(^3\)H-thymidine was added overnight. A stimulation index of \(\geq 3\) was considered to indicate a positive lymphoproliferative response.

**Magnetic Cell Sorting (MACS)**

For MACS separation (Miltenyi Biotec, Bergisch Gladbach, Germany), isolated MNC from anti coagulated human blood were labelled with MACS CD4 or CD8 MicroBeads, incubated for 15 minutes at 8°C, and washed extensively. Thereafter, cell suspensions were passed through a positive selection column type MS\(^+\) which was placed in the magnetic field of a MiniMACS separator. After removal of the column from the magnetic field, the magnetically retained CD4\(^+\) or CD8\(^+\) cells could be eluted as positively selected cell fraction. The unlabelled cell fraction was depleted of CD4\(^+\) and CD8\(^+\) cells.

**Cytokine determination**

PBMNC (10\(^5\) / 200 µl), *A. fumigatus* antigen EC SAB (5µg/ml) or the 90 kDa catalase (5µg/ml) were cultured in 96-well, round-bottomed plates. After 5 days of culture, the supernatant was removed from each well and stored at -80°C. Supernatants were tested for IL-10 and IFN-\(\gamma\) by use of commercial antigen-capture ELISA kits (IL-10-ELISA, DPC Biermann; IFN-\(\gamma\)-ELISA, Biozol). In case of cytokine concentrations below the analytical sensitivity of the assay, a concentration of 1 pg/ml for the respective cytokines was used to calculate the IFN-\(\gamma\)/IL-10 ratio.

**Tumor necrosis factor (TNF) bioassay**

To assess non-peptide specific T cell reactivities of well characterized \(V\gamma9/V\delta2\) phosphoantigen-reactive T-cell clones (19), a TNF bioassay was performed as described previously using the ultra-sensitive WEHI-164 clone 13 (20). The WEHI cells were grown in
complete medium supplemented with 5% FCS (Gibco). All assays were calibrated with recombinant human TNF.

Patients

*A. fumigatus*-specific T cell reactivities were assessed in adult healthy individuals, and in patients with clinical evidence of invasive aspergillosis after dose-intensive induction/consolidation chemotherapy for acute myeloid (n=8) and acute lymphoblastic leukemia (n=4), as well as in patients after allogeneic peripheral blood SCT from matched sibling donors (n=4) and after bone marrow transplantation from a matched unrelated donor (n=4) (Table 1). The median age in patients treated with chemotherapy only was 46 [29-67] and in patients after allogeneic SCT 35.5 [26-48] years. Myeloablative conditioning therapy prior to allogeneic SCT consisted either of fractionated total body irradiation (12 Gy) or busulfan (16 mg/kg body weight (bw)), both in combination with cyclophosphamide (120 mg/kg bw). Patients with high-risk leukemia were additionally treated with etoposide (40 mg/kg bw) or cytosinarabinoside (2x 2g/m² on 2 successive days). Graft-versus-host disease prophylaxis consisted of cyclosporine A according to serum levels and antithymocyte globuline for 3 days at 20 mg/kg bw on days –4 to –2 in patients receiving a transplant from a matched sibling donor, or for 4 days (-4 to –1) in patients receiving a transplant from a matched unrelated donor.

In order to compare the immune reconstitution to fungal, bacterial, and viral antigens late after allogeneic SCT, blood samples from 18 patients taken at a median of 134 [86-468] days after transplantation were analyzed. Patients characteristics are shown in detail in Table 2. This time point was selected, as according to our previous findings, CMV-specific lymphoproliferative responses can be demonstrated in the majority of patients at around day 100 post-transplantation (18). All patients gave informed consent to donate blood for immune reconstitution studies.
Definition of Invasive Aspergillosis

Invasive aspergillosis was categorized according to consensus criteria recently published by the European Organization for Research and Treatment of Cancer / Invasive Fungal Infections Co-operative Group (EORTC/IFICG) and the National Institute of Allergy and Infectious Diseases / Mycoses Study Group (NIAID/MSG) (21).

A diagnosis of a proven invasive aspergillosis required histological proof of a mould infection and/or a positive culture obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with infection.

Diagnosis of probable invasive aspergillosis required at least one defined host factor (neutropenia <500/µl for >10 days, fever for >96h refractory to broad spectrum antibiotics, signs and symptoms of graft-versus-host disease (GvHD) or prolonged use of corticosteroids), and at least one microbiological (positive culture from sputum or bronchoalveolar lavage fluid, positive cytology or microscopy from a sinus aspirate) and major clinical criterion (new pulmonary infiltrates, halo sign, air-crescent sign, suggestive radiological findings for an invasive sinus or central nervous system infection).

Possible invasive aspergillosis was defined as at least one criterion from the host section and one microbiological or one major clinical criterion from an abnormal site consistent with infection (see above).

According to the state of the lung infiltrates in the CT scan at the time of blood sampling, patients were classified to suffer from “regression” of IA if the lesions were smaller compared to previous CT-scans, “stable” if there was no difference in the extent of disease manifestations at both dates of examination, or “progression” if the manifestations of IA became worse compared to the preceding assessment.

Statistical analysis
All observed variables were markedly non-normally distributed and therefore described with their median and ranges. Wilcoxon signed-ranks tests for dependent variables were applied to compare the stimulation indices of the best concentration of EC SAB for lymphoproliferation with the stimulation indices of the other EC SAB concentrations assessed in healthy volunteers. Mann-Whitney U tests were conducted to compare the distributions of the variables between patients with progressive clinical manifestations of invasive aspergillosis and patients with clinical response upon antifungal treatment. Spearman’s rank-order correlation coefficients were computed to describe association between Aspergillus-specific lymphoproliferation and neutrophil counts and steroid doses and between steroid doses and IFN-γ and IL-10 release in culture supernatants. McNemar’s tests and sign tests were used to compare the lymphoproliferative responses after stimulation with CMV, EC SAB and tetanus toxoid, and to compare INF-γ/IL-10 ratios. All statistical analyses in this study were done for descriptive purposes without pre-stated hypotheses. Test results were presented with nominal two-tailed p-values. All analyses were carried out with JMP version 3.1.6.2 and SAS system for Windows 8.0 software (SAS Institute, Cary, NC).

Results

Lymphoproliferative responses to A. fumigatus antigens in healthy individuals

Mononuclear cells from healthy volunteers (n=16) were incubated with declining concentrations of A. fumigatus total antigenic extract EC SAB ranging from 50 µg to 50 ng equivalent protein/ml. A maximum stimulation index (SI) of ≥ 3 [median SI 7.1, range 2.1 – 86.9] was documented in 14 of 16 healthy individuals (87.5%). All healthy individuals demonstrated a significant lymphoproliferation to PHA [median SI 15.6, range 4.1 – 176.5] and tetanus toxoid [median SI 9.4, range 3.3 – 191.6], and 14 of 16 to IL-2 [median SI 11, range 2.2 – 132.5]. Lymphocytes stimulated with 5 µg/ml of EC SAB antigen demonstrated 3.3, 1.5 and 2.5-fold greater proliferation than did those stimulated with 50 µg/ml, 500 ng/ml
or 50 ng/ml (5 vs 50 µg, P<0.0001; 5 vs 0.5 µg, P=0.013; 5 vs 0.05 µg, P<0.0001). On the basis of these results, we selected the 5 µg/ml concentration for all further experiments.

The lymphoproliferative capacity of CD4+, CD8+ and CD4-/CD8- T cell subpopulations was assessed after stimulation with EC SAB in 3 individuals. In all cases, the CD4+ T cell fraction was found to proliferate in response to EC SAB, whereas no significant lymphoproliferation was found in the CD8+ and CD4-/CD8- lymphocyte populations (data not shown).

Lymphoproliferative responses to heat-inactivated conidia were assessed in 8 healthy individuals with 7 out of 8 (87.5%) demonstrating a significant lymphoproliferation [median SI 17.45; range 1.6 – 83.1]. The best lymphoproliferative response was documented at a concentration of 1x 10⁵ Aspergillus conidia/ml. Like with EC SAB the stimulation was definitely lower with the highest dose tested.

A significant lymphoproliferative response to the recombinant antigens was documented in 8 of 11 healthy individuals after stimulation with the 90 kDa catalase, and in 4 of 16 individuals in response to the 88 kDa dipeptidylpeptidase V (Figure 1).

**Cytokine secretion in culture supernatants of healthy individuals**

Peripheral blood mononuclear cells from 17 healthy individuals were stimulated for 5 days with the A. fumigatus antigen EC SAB at a concentration of 5 µg/ml, and with the 90 kDa catalase in 11 out of these 17 individuals.

The baseline IFN-γ and IL-10 release in culture supernatants after 5 days of culture without the addition of antigen was assessed in 6/17 healthy individuals. IFN-γ was detectable at a median concentration of 18.1 [range <1.0-83.8] pg/ml and IL-10 at a median concentration of 1.2 [range <1.0–11.9] pg/ml.

After stimulation of PBMNCs with EC SAB, an increase in the production of IFN-γ [median 128.7; range 21.3-1519 pg/ml] and IL-10 [median 39.4; range 1.5–899.4 pg/ml] was documented, with 13/17 demonstrating IFN-γ concentrations at least 2x higher compared to
IL-10. The cytokine release in culture supernatants after stimulation with the 90 kDa catalase revealed comparable results with an increased secretion of IFN-γ [n=11, median concentration 149.3; range 14.4->2000 pg/ml] and IL-10 [n=10, median concentration 10.05; range 3.6–168.5 pg/ml] (Figure 2).

\[\text{V\gamma9/V\delta2 T-cell clones are reactive to Aspergillus fumigatus antigens}\]

\[A. fumigatus\]-specific responses of well characterized \(V\gamma9/V\delta2\) T cell clones were assessed by a TNF bioassay. In 6 independent experiments, well characterized phosphoantigen-reactive \(V\gamma9/V\delta2\) T-cell clones, but not control \(\alpha/\beta\) and control phosphoantigen-non reactive \(\gamma/\delta\) T cell clones, produced significant quantities of TNF (20 to 40 pg/ml) to the ECSAB antigen (dilution 1/40), suggesting that \textit{Aspergillus} antigen-preparations may contain non-peptidic antigens (Figure 3).

\[\text{Aspergillus-specific T cell responses in patients with clinical evidence of invasive aspergillosis}\]

According to the above-mentioned definitions, 5 patients suffered from proven, 3 from probable, and 12 from possible invasive aspergillosis.

After stimulation with the whole \textit{Aspergillus fumigatus} antigen extract EC SAB, a positive lymphoproliferative response was documented in 14/18 patients [median SI 9.05, range 1.0-41.4] (Table 1). Two additional patients (#7, #20) not tested with EC SAB showed a positive lymphoproliferative response to an ethanol precipitate of a culture filtrate of \textit{Aspergillus fumigatus} (PP EXL) grown in a culture medium containing 1% Yeast extract (Difco, Detroit) (SI 3.6 and 13.2 respectively). A positive lymphoproliferative response to PHA was documented in 14 out of these 20 patients [median SI 18.75, range 0.6-332.1], to tetanus toxoid in 10 out of 18 analyzed [median SI 3.6, range 0.7-27.2], and in 17 out of 20 patients to IL-2 [median SI 15.95, range 0.5-201.5].
Proven or probable invasive aspergillosis was diagnosed in 5 patients after allogeneic SCT (#1, #2, #6 - #8), and in 3 further patients following intensive chemotherapy for acute myeloid leukemia (#3 - #5) the IA was proven (Table 1). Six out of 7 patients showed a positive lymphoproliferative response to EC SAB and patient #7 to the ethanol precipitate. Five out of these 8 patients demonstrated at least a partial regression of clinical manifestations of IA, and one patient after allogeneic SCT survived cerebral aspergillosis for >6 weeks (#6) (Table 1). After initial improvement and control of invasive aspergillosis, patients #4 and #5 underwent an allogeneic SCT and subsequently died from disseminated IA. Another 12 patients with possible invasive aspergillosis according to the above-mentioned definitions were analyzed and 8/11 showed a SI ≥3 in response to EC SAB and patient #20 after stimulation with the ethanol precipitate (Table 1). A low stimulation index in response to the EC SAB antigen was demonstrated in 3 out of 6 patients after allogeneic SCT with progressive disease (Table 1, #9-#11).

The release of IFN-γ and IL-10 in culture supernatants after stimulation with EC SAB was assessed in 17 patients. Baseline cytokine release in culture supernatants after 5 days of culture assessed in 6 patients demonstrated low concentrations of IFN-γ [median 2.4; range <1.0-20.8 pg/ml] and IL-10 [median 1.6; range <1.0–11.9 pg/ml] [median ratio IFN-γ/IL-10 0.9; range 0.2-21]. A higher ratio of IFN-γ/IL-10 was documented in patients with favourable response to antifungal therapy (n=7, median ratio IFN-γ/IL-10=1.0; range 0.09–24.8) compared to 10 patients with progressive (n=6) or stable (n=4) disease (median ratio IFN-γ/IL-10 = 0.1; range 0.002–2.1) (P=0.04). Interestingly, 2 out of the 7 patients that demonstrated a favourable response to antifungal therapy at the time of analysis despite a low Aspergillus-specific lymphoproliferation or a low IFN-γ/IL-10 ratio (Table 1, #2 and #8, Figure 4), finally died from disseminated invasive aspergillosis within 3 – 14 months. Both patients received prolonged immunosuppression following allogeneic SCT. Thus, control of
invasive aspergillosis was found to be correlated with a Th1 response (Figure 4, patient #1), whereas fatal outcome of the infection was associated with a low stimulation index (Figure 4, patient #2) and a Th2 cytokine pattern (Figure 4, patients #8 and #11).

The correlation of the clinical response at the time of analysis revealed *A. fumigatus*-specific lymphoproliferation to be correlated with the neutrophil count (R=0.459, P=0.064), and to be inversely correlated with the steroid dose (R=-0.508, P=0.037). Moreover, steroid treatment was associated with suppression of IFN-γ (R=0.572, P=0.017) but not IL-10 release (R=-0.2543, P=0.325) in culture supernatants.

Comparative analysis of lymphoproliferative responses to *Aspergillus fumigatus*, tetanus toxoid and cytomegalovirus in patients late after allogeneic stem cell transplantation

In order to compare the immune reconstitution to fungal, bacterial and viral antigens in patients after allogeneic SCT, blood samples from 18 patients taken at a median of 134 [86-468] days after SCT were analyzed. This time point was selected as, according to our previous results, CMV-specific T-cell reconstitution can be demonstrated in the majority of patients at around day 100 post-transplantation.

A positive lymphoproliferative response was documented in 11/14 patients - tested CMV-seropositive pretransplant and/or receiving a transplant from a CMV-seropositive donor - after stimulation with CMV antigen (median SI 8.0; range 1.0 – 53.8), compared to 4/18 patients after stimulation with the *Aspergillus fumigatus* antigen EC SAB (median SI 1.2; range 1.0 – 37.6) (P = 0.008, McNemar’s test), and to 3/18 after stimulation with tetanus toxoid (median SI 1.15; range 1.0 – 6.9) (P = 0.003, McNemar’s test) (Figure 5A). No lymphoproliferative response to CMV antigen was detectable in 4 CMV-seronegative patients receiving a transplant from a CMV-seronegative donor (data not shown). Interestingly, 3 out of the 4 patients demonstrating an *Aspergillus*-specific lymphoproliferation suffered from
invasive aspergillosis after transplantation (n=2) or had a positive history of invasive aspergillosis pre-transplantation (n=1).

The cytokine concentrations of IFN-γ and IL-10 were analyzed after stimulation with EC SAB and tetanus toxoid in 17 patients, and after stimulation with CMV antigen in 13 patients. The median IFN-γ/IL-10 ratio in response to tetanus toxoid (median ratio 4.04; range 0.026 – 202) was higher compared to the stimulation with EC SAB (median ratio 0.28; range 0.006 – 2.93, P = 0.002, sign test) and CMV antigen (median ratio 0.58; range 0.003 – 95.24, P = 0.267, sign test) (Figure 5B). Thus, the *Aspergillus*-specific T-cell response in patients late after allogeneic SCT was characterized by a low stimulation index and a low IFN-γ/IL-10 ratio, indicating a T_h2 response in some of the patients, potentially contributing to the prolonged risk for the development of invasive aspergillosis in this patient group.

**Discussion**

In the last 1 to 2 decades, an important change in the epidemiology of invasive aspergillosis was observed. Whereas in earlier years, this devastating disease was almost always observed in hematological patients with long-lasting neutropenia, invasive aspergillosis was reported more recently with an increasing frequency in non-neutropenic patients after allogeneic SCT (1), in patients with advanced HIV infection (6), and in critically ill neonates (7). Transmission via airborne spores was identified as the major route of infection (22). Some patients are obviously colonized without developing invasive disease, in others, the infection remains restricted to the lungs or may disseminate to a variety of organs and tissues, and upon post-mortem analysis, *Aspergillus* may be documented in almost all tissues (23).

The pathophysiology of invasive aspergillosis, especially in non-neutropenic patients is not completely understood. Well recognized risk factors for invasive aspergillosis are defects in phagocyte function (2), corticosteroid-induced suppression of phagocyte function (3, 24), and long-lasting neutropenia (4). More recently, an increased incidence of invasive fungal
infections was observed in patients after allogeneic bone marrow compared to peripheral blood SCT (25). The only difference between both groups was a faster T cell reconstitution after transplantation of peripheral blood stem cells indicating a potential role of T cells for the control of fungal infections.

Dysregulation of cytokine release has been identified as an important risk factor in patients with HIV infection (26). In the murine model of invasive pulmonary aspergillosis, resistance to infection was associated with IFN-γ producing interstitial lung lymphocytes, and TNF-α (10, 12, 13) and IL-12 production (12), whereas a dominant release of TH2 cytokines of interstitial lung lymphocytes was associated with progressive disease. TH1-mediated resistance to invasive aspergillosis was further confirmed by neutralization of TH2 cytokines and in IL-4 and IL-10 knockout animals (12, 27). Protective immunity was documented in animals sensitized with a sublethal challenge of A. fumigatus conidia developing a TH1 response upon subsequent exposure to lethal infection (10, 12). Treatment of immunocompetent mice with Aspergillus crude culture filtrate antigens resulted in the development of local and peripheral protective TH1 memory responses, mediated by Aspergillus-specific CD4+ T cells producing IFN-γ and IL-2 capable of conferring protection upon adoptive transfer to naïve recipients (15).

Based on these clinical observations and experimental results, A. fumigatus-specific T cell responses were assessed in healthy individuals, immunosuppressed patients with clinical evidence of invasive aspergillosis, and non-neutropenic patients late (>100 days) after allogeneic SCT. Almost all healthy individuals demonstrated a significant lymphoproliferation to cellular extracts of A. fumigatus, heat-inactivated A. fumigatus conidia as reported before (28), and to the 2 major antigens of A. fumigatus, the 90 kDa catalase and the 88 kDa dipeptidylpeptidase V. Assessment of the IFN-γ and IL-10 concentrations in culture supernatants after specific stimulation suggested a TH1 type of immune response. Patients with clinical evidence of invasive aspergillosis and disease regression upon
antifungal therapy were characterized by positive *A. fumigatus*-specific lymphoproliferation and a higher ratio of IFN-γ/IL10 in culture supernatants after specific stimulation with cellular extracts of *A. fumigatus* compared to patients with stable or progressive disease demonstrating a lower IFN-γ/IL10 ratio in culture supernatants (P=0.04). Interestingly, 2 out of the 7 patients that initially demonstrated a favourable response to antifungal therapy despite a low *Aspergillus*-specific lymphoproliferation or a low IFN-γ/IL-10 ratio finally died from disseminated invasive aspergillosis. Both patients had received prolonged immunosuppression after allogeneic SCT. Thus, in line with the results of the murine model of invasive aspergillosis, our data further support a potential role of a Th1 immune response for the successful control of invasive aspergillosis in patients with hematological malignancies.

Major risk factors for invasive aspergillosis after allogeneic SCT are prolonged neutropenia, acute and chronic graft-versus-host disease and corticosteroid treatment (1). In our study, a low lymphoproliferative response to *A. fumigatus* antigens was found to be associated with low neutrophil counts, corticosteroid treatment, and a low release of IFN-γ in culture supernatants upon specific stimulation. Patients with progressive disease despite a positive lymphoproliferative response to *Aspergillus* antigens either demonstrated a low ratio of IFN-γ/IL10 in culture supernatants or very low levels of both cytokines. Thus, we conclude from this analysis suppression of *A. fumigatus*-specific Th1 response after allogeneic SCT during GvHD and corticosteroid treatment to be a predisposing factor for the acquisition and progression of invasive aspergillosis. In line with these results, non-neutropenic patients with favourable outcome demonstrated low IL-10 levels, whereas progressive disease was associated with elevated IL-10 levels (29). Moreover, we found *Aspergillus*-specific T-cell responses to be characterized by a low stimulation index and a low IFN-γ/IL-10 ratio in many
patients late after allogeneic SCT, potentially contributing to the prolonged risk for the development of invasive aspergillosis in this patient group (1).

In addition to T helper cell responses, the release of TNF from γ/δ T-cell clones from healthy individuals in response to *A. fumigatus* was studied. Phosphoantigen-reactive Vγ9/Vδ2 T-cell clones, but not control α/β and control phosphoantigen-non reactive γ/δ T-cell clones, produced significant quantities of TNF to the EC SAB antigen. These data indicate that *Aspergillus* antigen-preparations may contain non-peptidic antigens for Vγ9/Vδ2 T cells (30-32). To our knowledge, aspergillus antigens have not been described up to now to stimulate γ/δ T-cells. Our data suggest that Vγ9/Vδ2 T cells may contribute to the protective immune response against invasive aspergillosis. Early expansion of CD45RO+ Vγ9/Vδ2 T cells during the first weeks post-transplantation, possibly on contact with environmental antigens, has been reported in patients after allogeneic SCT (33), and supranormal levels of γ/δ T cells were found to be associated with infectious complications after allogeneic SCT (34). Thus, γ/δ T cells may function as a bridge between innate and adaptive immunity in the defense against fungal infections (35).

In conclusion, the data reported support the hypothesis that in patients with hematological malignancies, T-cells may contribute to the host defense against *A. fumigatus*. In the future, a detailed analysis of *Aspergillus*-specific T-cell responses may help to develop new antifungal treatment strategies, such as treatment with proinflammatory cytokines, inhibition of IL-10 or the adoptive transfer of *A. fumigatus*-specific T_H1 cells in patients after allogeneic SCT.

**Acknowledgments**

We thank Prof. Dr. Hans-Georg Rammensee and Prof. Dr. Lothar Kanz for helpful discussions, and Friederike Frank for technical assistance.
References


33. van der Harst D, Brand A, van Luxemburg-Heijs SA, Kooij-Winkelaar YM, Zwaan FE, Koning F. Selective outgrowth of CD45RO+ V gamma 9+/V delta 2+ T-cell


Figure legends

**Figure 1:** *Aspergillus*-specific T cell responses in healthy volunteers.

*Aspergillus*-specific T cell responses were assessed by a lymphoproliferation assay. A stimulation index of $\geq 3$ was considered to indicate a positive lymphoproliferative T cell response. *Aspergillus*-specific T cell proliferation was detectable against EC SAB in 14 of 16 healthy individuals, against heat-inactivated conidia in 7 of 8, and against 2 recombinant *A. fumigatus* proteins expressed in *Pichia pastoris*, the 90 kDa catalase in 8 of 11 and the 88 kDa dipeptidylpeptidase V in 4 of 16 respectively.

EC SAB, cellular extracts of *A. fumigatus*; Cat19, 90 kDa catalase; DPP, dipeptidylpeptidase

**Figure 2:** *Aspergillus*-specific T cell stimulation in healthy volunteers is associated with increased production of IFN-$\gamma$ and IL-10 in culture supernatants.

Cytokines were measured by standard ELISA assays in the culture supernatants of PBMNC’s stimulated with *A. fumigatus* proteins. After stimulation of PBMNCs with EC SAB, an increase in the production of IFN-$\gamma$ [median 128.7; range 21.3–1519 pg/ml] and IL-10 [median 39.4; range 1.5–899.4 pg/ml] was documented. The cytokine release in culture supernatants after stimulation with the 90 kDa catalase revealed comparable results [median IFN-$\gamma$ concentration 149.3; range 14.4–2000 pg/ml, median IL-10 concentration 10.05; range 3.6–168.5 pg/ml].

EC SAB, cellular extracts of *A. fumigatus*; Cat19, 90 kDa catalase

∇IFN-$\gamma$, ∆IL-10,

*IL-10 concentrations were assessed in 10/11 healthy individuals*
Figure 3: Phosphoantigen-reactive Vγ9/Vδ2 T-cell clones, but not α/β and phosphoantigen-non reactive γ/δ T-cell clones, produce TNF in response to Aspergillus fumigatus antigens.

Aspergillus-specific T cell responses were assessed by a TNF bioassay using the ultra-sensitive WEHI-164 clone 13. The phosphoantigen-reactive Vγ9/Vδ2 T-cell clones C49 and 6E1, but not the phosphoantigen-non reactive γ/δ T-cell clone AB18 and the α/β T-cell clone B1, produced significant amounts of TNF in response to Aspergillus fumigatus antigen EC SAB.

Figure 4: Longitudinal analysis of Aspergillus-specific T-cell responses in 4 patients with invasive aspergillosis after allogeneic SCT.

Long term control of invasive aspergillosis was associated with a lymphoproliferative response to EC SAB and a dominant release of IFN-γ (patient #1), whereas a low stimulation index (patient #2) and/or a dominant release of IL-10 (patients #8 and #11) was found to be associated with disease dissemination and fatal outcome of invasive aspergillosis after allogeneic SCT.

-○-, stimulation index; □, IFN-γ; ▣, IL-10

Figure 5: Comparative analysis of Aspergillus-, tetanus toxoid- and CMV antigen-specific T-cell responses in patients late after allogeneic SCT

Cytokines were measured by standard ELISA assays in the culture supernatants of PBMNC’s stimulated with A. fumigatus protein EC SAB. Panel A shows the stimulation indices, Panel B the ratio of IFN-γ and IL-10. A stimulation index of ≥3 was considered to indicate a positive lymphoproliferative T cell response. Aspergillus-specific T-cell responses in patients late after allogeneic SCT were characterized by a low stimulation index and a low IFN-γ/IL-10 ratio.
Figure 1
Figure 2
Figure 3
### Table 1: Patient Data

<table>
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<tr>
<th>Patient</th>
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<th>Steroids [mg/kg]</th>
<th>Pulmonary Infiltrates</th>
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### Figure 4

![Graphs showing cytokine concentrations and stimulation index over days after transplantation](#)
Figure 5
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<th>Treatment</th>
<th>GvHD</th>
<th>Pred</th>
<th>Neutropenia</th>
<th>SI</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Ratio</th>
<th>Clinical sites of IA</th>
<th>Aspergillus species</th>
<th>pathology</th>
<th>Response#</th>
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<td>-</td>
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<td>Regression</td>
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<td>PBSCT</td>
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<td>lung</td>
<td>A. flavus (biopsy)</td>
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<td>-</td>
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<td>GvHD modality</td>
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<td>SI</td>
<td>IFN-γ [pg/ml]</td>
<td>IL-10 [pg/ml]</td>
<td>Ratio IFN-γ/IL-10</td>
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<td>Regression</td>
<td></td>
</tr>
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</table>

Patients with possible invasive aspergillosis

<p>| #9   | CML     | MUD       | °II           | 0.2         | 20                     | 2.3 | &lt;1           | 174.4         | &lt;0.006          | lung                   | -                         | Progression |
| #10  | CML     | MUD       | °IV           | 3.6         | 9                      | 3.0 | 22.3         | 313           | 0.07            | lung                   | -                         | Progression |
| #11  | CML     | MUD       | °II           | 0.6         | 7                      | 1.0 | &lt;1           | 55.6          | &lt;0.018          | lung                   | -                         | Progression |
| #12  | AML     | Chemo     |               | -           | -                      | 15  | 14.3         | &lt;1            | 607             | lung, cerebral        | -                         | Stable      |
| #13  | AML     | Chemo     |               | 1.6         | 41                     | 3.1 | &lt;1           | &lt;1            | -               | lung                   | -                         | Regression  |</p>
<table>
<thead>
<tr>
<th>Pat.</th>
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<th>Treatment</th>
<th>GvHD</th>
<th>Pred</th>
<th>Neutropenia</th>
<th>SI</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Ratio</th>
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**Table 1:** Clinical characteristics of patients with evidence of invasive aspergillosis

# status of invasive aspergillosis at the time of analysis

§ after initial improvement of IA, patients #4 and #5 underwent an allogeneic SCT and finally died from invasive aspergillosis

IA, invasive aspergillosis; GvHD, graft-versus-host disease; Pred., prednisolone; SI, stimulation index in response to EC SAB; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; allo PBSCT, allogeneic peripheral blood stem cell transplantation from an HLA-identical sibling donor; MUD-BMT, bone marrow transplantation from an HLA-matched unrelated donor; ext., extensive; BAL, bronchoalveolar lavage.
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Table 2: Clinical characteristics of patients analyzed late after allogeneic SCT

*CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; NHL, Non-Hodgkin’s lymphoma; MDS, myelodysplastic syndrome; HCMV, human cytomegalovirus; TBI, total body irradiation; CY, cyclophosphamide; VP16, etoposide; BU, busulfan; FLU, fludarabine; ARA-C, cytosinarabinoside; GvHD, graft-versus-host disease.*
Analysis of T-Cell Responses to Aspergillus Fumigatus Antigens in Healthy Individuals and Patients with Hematological Malignancies

Holger Hebart, Claudia Bollinger, Paul Fisch, Jacqueline Sarfati, Christoph Meisner, Manuela Baur, Juergen Loeffler, Michel Monod, Jean-Paul Latge and Hermann Einsele