Brief report

# Polymorphisms in the chemokine (C-X-C motif) ligand 10 are associated with invasive aspergillosis after allogeneic stem-cell transplantation and influence *CXCL10* epression in monocyte-derived dendritic cells

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**Patients after allogeneic stem-cell transplantation (alloSCT) have an increased risk for invasive aspergillosis (IA). Here, recipients of an allograft with IA (n = 81) or without IA (n = 58) were screened for 84 single nucleotide polymorphisms in 18 immune relevant genes. We found 3 markers in *chemokine (C-X-C motif) ligand* 10 (*CXCL10*, 4q21, 11 101 C > T, *P* = .007; 1642 C < G, *P* = .003; −1101 A < G, *P* = .001) significantly associated with an increased risk of developing IA. Furthermore, immature dendritic cells (iDCs) exposed to *Aspergillus fumigatus* germlings showed markedly higher *CXCL10* expression, if carrying the wild type genotype, compared with the "CGAG" high risk haplotype. In addition, serum from patients with proven/probable IA showed increased serum levels of CXCL10, compared with immunocompromised patients without IA. Thus, polymorphisms in *CXCL10* determine chemokine secretion by iDCs upon exposure to *A fumigatus* and most likely thereby genetically determine the risk of IA after alloSCT. (Blood. 2008;111:534-536)**



## Introduction

Infections with *Aspergillus fumigatus* are frequent and life threatening in patients after allogeneic stem-cell transplantation (alloSCT).[1] Several single nucleotide polymorphisms (SNPs) were described influencing the course and outcome of invasive aspergillosis (IA). Kesh and colleagues revealed an association of IA with polymorphisms in Toll-like receptor genes *TLR1* and *TLR6*, whereas no association could be found for the *TLR4* gene.[2] In addition, it has been shown that polymorphisms in *mannan-binding lectin* (*MBL*) contribute to the occurrence of allergic bronchopulmonary aspergillosis (ABPA) by influencing the MBL plasma level and protein activity.[3] Besides *MBL*, further SNPs in C-type lectins were investigated leading to the identification of an association between ABPA and variants of the *surfactant protein A2 gene* (*SFPTPA2*).[4] In contrast, alleles with a protective role in the pathogenesis of IA were discovered in the promoter region of *IL10*.[5]

## Methods

This study was approved by the local ethics committees involved in the study (University of Innsbruck, Innsbruck, Austria; University of Graz, Graz, Austria; Karolinska University Hospital, Stockholm, Sweden; and Medical Hospital II, Tübingen, Germany).

### Patient and sampling data

After approval of the local ethics committees, each consecutive patient who underwent alloSCT and who showed complete donor chimerism at the time of blood collection (as determined by short tandem repeat markers[6]) was asked for study participation. After informed consent was obtained in accordance with the Declaration of Helsinki, blood samples from 139 consecutive patients were collected between day +20 and day +50 after alloSCT. Patients suffered from acute myeloid leukemia (n = 57), chronic myeloid leukemia (n = 26), acute lymphatic leukemia (n = 20), multiple myeloma (n = 11), and other hematologic disorders (n = 25). Sampling was performed at the University of Innsbruck, Innsbruck, Austria (n = 16), University of Graz, Graz, Austria (n = 26), Karolinska University Hospital, Stockholm, Sweden (n = 13), and Medical Hospital II, Tübingen, Germany (n = 84). All patients were of European origin (median age 37y, range [7-61y], 82/139 males). Patients after alloSCT either developed proven or probable IA (as defined by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC-IFICG)/ National Institute of Allergy and Infectious Diseases/Mycoses Study Group (NIAID/MSG),[7] n = 81) whereas controls (alloSCT patients without IA, n = 58) did not fulfil these criteria.

According to a case-control study design, samples were analyzed for association between an increased risk for IA and defined genetic markers (n = 84) in 18 genes (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Genes were chosen for being differentially regulated in genome-wide expression profiling studies in cocultures of *A fumigatus* with immature dendritic cells (iDCs) or for being generally relevant for regulation of immune defense mechanisms. Retrospective genotyping was carried out as previously described on DNA from each patient who had given consent.[8]

The following clinical risk factors were tested for an association to IA: (1) selection of $CD34^+$ cells before alloSCT, (2) corticosteroid therapy with a

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dosage of more than 2 mg/kg body weight, (3) severe acute graft-versus-host disease (aGVHD), grade II – IV, (4) cytomegalovirus disease (HCMV), and (5) respiratory virus (RV) infection. HCMV disease was defined by Ljungman et al,[9] RV infection was defined as influenza A, influenza B, human parainfluenza 1-3, adenovirus, and respiratory syncytial virus detection by culture or reverse transcriptase–polymerase chain reaction (RT-PCR) assay.[10]

In a preliminary, add-on study, 1 mL serum from further consecutive patients after alloSCT, (each patient who agreed to participate was included, n = 33, median age 52 years, range [23-64 years], 19/33 males) and from randomly selected laboratory workers (n = 14, median age 28 years, range [23-41 years], 6/14 males) was collected for quantification of CXCL10 levels by ELISA (R&D Systems, Wiesbaden, Germany). Patients suffered from proven or probable IA (6/33) or showed no clinical signs of infection (27/33).

### Statistical analysis

Allele and genotype frequencies were compared between patients with IA and controls using Allele-Frequency-Difference-Test and Armitage Trend Test, respectively. *P* values less than .01 were considered to be significant. All markers were tested for Hardy-Weinberg equilibrium using the chi square test statistics with one degree of freedom, or an exact test in case of low expectation values (< 5). For 4 markers in *CXCL10*, haplotype analysis was performed with the program FAMHAP[8,11] (version 16; Dr Tim Beder, University of Bonn, Institut für Medizinische Biometrie, Informatik und Epidemiologie, Bonn, Germany) and the overall significance for the haplotype block was calculated based on a permutation based procedure to correct for multiples testing. CXCL10 serum levels were analyzed with the Wilcoxon test.

### Analysis of *CXCL10* expression upon stimulation with *A fumigatus*

Isolation of monocytes (obtained from the laboratory workers mentioned above) and differentiation into iDCs was achieved as described before.[12] Cocultivation of *A fumigatus* germlings (ATCC 9197) with iDCs was carried out for 5hours. *CXCL10* expression was analyzed by real-time PCR assay[13] using the following primers (F: acgtgttgagatcattgctacaa, R: gattttgctcccctctggt) and probes (P1: agtaaattcttgatggccttcgattct, P2: gattcagacatctcttctcacccttctttt, TIB MOLBIOL, Berlin, Germany). Results were normalized against the housekeeping gene 5-aminolevulinate synthase.[14]

**Table 1. Genotyped polymorphisms in *CXCL10* and statistical analysis**

| dbSNP number, allele | Control | IA | *P* |
|---|---|---|---|
| **rs1554013 (+11 101 C/T)** | | | .007* |
| C/C | 9 | 24 | |
| C/T | 29 | 20 | |
| T/T | 11 | 7 | |
| **rs3921 (+1642 C/G)** | | | .003* |
| C/C | 9 | 3 | |
| C/G | 27 | 15 | |
| G/G | 10 | 21 | |
| **rs4859588 (+908 A/G)** | | | .057 |
| A/A | 8 | 19 | |
| A/G | 28 | 20 | |
| G/G | 10 | 8 | |
| **rs4257674 (-1101 A/G)** | | | .001* |
| A/A | 12 | 5 | |
| A/G | 22 | 19 | . |
| G/G | 10 | 28 | |

Association analysis between genetic polymorphisms in *CXCL10* and the risk to develop IA for patients after alloSCT. Numbers of homozygous and heterozygous alleles used for statistical comparison are specified. Positions of the SNPs in the respective genes have been determined at http://snpper.chip.org/.

* indicates significant *P* values ($P < .01$) as obtained by Allele-Frequency Difference Test.

## Results and discussion

DNA specimens of 139 patients after alloSCT were screened for defined polymorphisms. The single marker analyses revealed no association with an increased risk for IA for 80 of the 84 genetic markers (Table S1). However, 3 SNPs in *CXCL10* (rs1554013, rs3921, and rs4257674[15]) and one marker in *IFN-γ* (rs2069705) showed a significant association with the occurrence of IA ($P < .01$, Table 1) .

Haplotype analysis for the 3 markers in *CXCL10* [rs1554013 (C/T), rs3921 (C/G), rs4859588 (A/G)] and the *IFN-γ* marker rs4257674 (A/G, located in a potential negative regulatory site) confirmed the single marker analysis and identified "CGAG" as the high risk haplotype (significance of the haplotype block: $P = .008$).

There is currently no consensus on how to adjust the level of significance in exploratory epidemiologic studies. The Bonferroni correction for multiple testing is often considered too conservative. In this study, *P* values less than .01 were considered to be significant for single marker analysis, but it cannot be excluded that even *P* values less than .05 can be regarded as indicators for an association with the occurrence of IA.[16,17] We believe that multiple occurrence of several markers with low ($P < .01$) *P* values in one gene region and a significant *P* value of the haplotype analysis corrected for multiple testing give strong evidence of a true association.

CXCL10 is an inflammatory mediator, induced by IFN-γ, which stimulates the directional migration of Th1 cells as well as increasing T-cell adhesion to endothelium.[17] Antigen-specific proliferation of T cells occurred in healthy individuals and in patients surviving IA, indicating that T-lymphocytes play a pivotal role in fungal clearance.[19] Furthermore, Th1/Th2 deregulation and a switch to Th2 immune response may contribute to an unfavorable outcome of IA.[19]

Serum levels of CXCL10 were compared in patients after alloSCT and in healthy individuals. In sera from patients who survived proven or probable IA (mean 998 pg/mL, range [753-1445 pg/mL], $P = .002$) as well as in specimens from patients without signs of infection (mean 554 pg/mL, range [151-1030 pg/mL], $P = .004$), CXCL10 levels were significantly higher compared with healthy controls (mean 102 pg/mL, range [28-372 pg/mL]).

To study the role of *A fumigatus* for *CXCL10* induction, *A fumigatus* germlings were cocultured with iDCs generated from blood of healthy volunteers. Stimulation with *A fumigatus* augmented *CXCL10* expression, dependent on the respective genoptype. In iDCs carrying the wild type alleles, *CXCL10* expression increased notably [53.4×-335.5×], whereas in iDCs with the identified risk haplotype, expression ranged between 1.5× and 35.8× only. In the mean, markedly higher mRNA levels (9.3×) could be detected in iDCs carrying the wild type haplotype. These observations confirm the relevance of the risk haplotype "CGAG" for CXCL10 production and support the role of CXCL10 for effective immune defense against *A fumigatus*.

Table 2 shows the odds ratios of the clinical risk factors analyzed. Although most of them are not statistically significant, they point in the expected direction of being associated with *A fumigatus* infection. We suppose that lower *P* values might have been found in larger patient cohorts, as demonstrated by Upton et al in 405 cases.[20] We found an especially strong association for the occurrence of RV infections ($P = .004$) with *A fumigatus* infection. This observation confirms data from Marr et al, who assumed that increased risk may be related to effects on pulmonary phagocyte function.[21]

Only a few reports exist about an association of SNPs with susceptibility to IA, including markers in mannose-binding lectin and TLR genes.[2,3,22] In contrast to Bochud et al, who showed that a distinct haplotype in *TLR4* increased the risk of mould infections,

**Table 2. Correlation between defined clinical parameters and patients with or without proven/probable IA after alloSCT**

| Clinical parameters | Patients with proven/probable IA, % | Controls, % | *P* | Odds ratio |
|---|---|---|---|---|
| **Corticosteroid therapy** | | | .971 | 0.98 [0.29-3.19] |
| Less than 2 mg/kg | 58.0 | 57.4 | | |
| More than 2 mg/kg | 42.0 | 42.6 | | |
| **Acute GVHD** | | | .161 | 2.11 [0.63-7.11] |
| Grade 0 to 1 | 52.6 | 70.4 | | |
| Grade 2 to 4 | 47.4 | 29.6 | | |
| **Chronic GVHD** | | | .367 | 2.31 [0.31-15.32] |
| None | 84.2 | 92.6 | | |
| Limited/extensive | 15.8 | 7.4 | | |
| **CD34 selection** | | | .144 | 0.24 [0.02-2.40] |
| Yes | 93.5 | 76.9 | | |
| No | 6.5 | 23.1 | | |
| **RSV infection** | | | .004* | 6.05* [1.65-23.68] |
| Yes | 52.6 | 15.1 | | |
| No | 47.4 | 84.9 | | |
| **CMV infection** | | | .112 | 2.25 [0.72-7.14] |
| Yes | 45.8 | 27.1 | | |
| No | 54.2 | 72.9 | | |

* indicates significant *P* value (<.01) and odds ratio.

our analyses did not show an association between polymorphisms in *TLR4* (n = 5) and the probability of IA.[23]

In conclusion, screening of patients after alloSCT for the presence of defined alleles in *CXCL10* might have an impact on early identification of patients at risk for the development of IA, and thus on individualization of antifungal prophylaxis and treatment. A prospective study, evaluating treatment / prophylaxis strategies based on genetic polymorphisms in *CXCL10,* is under development.

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## Authorship

Contribution: M.M. performed research, analyzed data, and wrote; the manuscript; M.S. and T.F.W. performed data analysis; M.B. performed research and data analysis; C.M.J.E. and M.R.T. performed research; P.L. and H.J.D. collected data; H.H. and H.E. designed research; and J.L. designed research, analyzed data, and wrote the manuscript.

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