The Y238X Stop Codon Polymorphism in the Human β-Glucan Receptor Dectin-1 and Susceptibility to Invasive Aspergillosis

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Background. Dectin-1 is the major receptor for fungal β-glucans on myeloid cells. We investigated whether defective Dectin-1 receptor function, because of the early stop codon polymorphism Y238X, enhances susceptibility to invasive aspergillosis (IA) in at-risk patients.

Methods. Association of Dectin-1 Y238X polymorphism with occurrence and clinical course of IA was evaluated in 71 patients who developed IA post hematopoietic stem cell transplantation (HSCT) and in another 21 non-HSCT patients with IA. The control group consisted of 108 patients who underwent HSCT. Functional studies were performed to investigate consequences of the Y238X Dectin-1 polymorphism.

Results. The Y238X allele frequency was higher in non-HSCT patients with IA (19.0% vs 6.9%–7.7%; \( P < .05 \)). Heterozygosity for Y238X polymorphism in HSCT recipients showed a trend toward IA susceptibility (odds ratio, 1.79; 95% CI, .77–4.19; \( P = .17 \)) but did not influence clinical course of IA. Functional assays revealed that although peripheral blood mononuclear cells with defective Dectin-1 function due to Y238X responded less efficiently to Aspergillus, corresponding macrophages showed adequate response to Aspergillus.

Conclusions. Dectin-1 Y238X heterozygosity has a limited influence on susceptibility to IA and may be important in susceptible non-HSCT patients. This is partly attributable to redundancy inherent in the innate immune system. Larger studies are needed to confirm these findings.

Invasive fungal infections remain a major cause of morbidity and mortality in immunocompromised patients; invasive aspergillosis (IA) is emerging as the most common invasive fungal infection [1, 2]. Susceptibility and host response to fungal infection is largely determined by the immune status of the host and its ability to recognize the pathogen and to respond appropriately [3]. The mechanism responsible for this recognition is represented by pattern recognition receptors (PRRs), which include the family of Toll-like receptors (TLR) and C-type lectin receptors [4]. Dectin-1 is a C-type lectin receptor present on human immune cells (eg, macrophages and monocytes). It recognizes the β-1,3-glucan motif present on the cell walls of Candida and Aspergillus species and mediates host immune response to these fungal pathogens [5].

Recently, we described a functional single nucleotide polymorphism (SNP) in Dectin-1 (Y238X, rs16910526) leading to an early stop codon, which resulted in loss of the last 10 amino acids of the carbohydrate-recognition domain of the Dectin-1 receptor. Subsequently, this resulted in diminished...
expression of the Dectin-1 receptor on immune cells and its inability to bind β-glucan, leading to defective production of proinflammatory cytokines [6]. Clinically, this polymorphism was found to be associated with colonization with Candida species in hematopoietic stem cell transplant (HSCT) recipients [7] and with recurrent mucocutaneous fungal infection in a Dutch family [6].

Results from in vitro and murine models have shown that Dectin-1 is pivotal in host defense against Aspergillus infection [8–10]. However, no data are available from human studies to validate these findings. Thus, we aimed to investigate the clinical relevance of the Dectin-1 early stop codon polymorphism for the susceptibility and outcome of IA in a cohort of patients with underlying hematological disorders.

PATIENTS AND METHODS

Patient Population

Ninety-two patients of Dutch-Flemish ancestry who had underlying hematological diseases and had received a diagnosis of IA between May 1996 to July 2009 were enrolled from 3 academic hospitals: Leiden University Medical Center, Radboud University Nijmegen Medical Center (both in the Netherlands), and University Hospitals Leuven (Belgium). Of these 92 patients, 71 developed IA after allogenic HSCT and 21 other patients had IA after receiving chemotherapy but without undergoing HSCT. Invasive aspergillosis had been diagnosed as either proven or probable IA according to current European Organization for Research and Treatment of Cancer/Mycology Table 2a. Incidence of Dectin-1 Y238X variant in HSCT patients/donors between IA cases and controls

<table>
<thead>
<tr>
<th>Study Cohort</th>
<th>IA Cases Dectin-1 Y238X variant present n/N</th>
<th>Controls Dectin-1 Y238X variant present n/N</th>
<th>Univariate OR (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSCT recipients with IA vs control recipients</td>
<td>13/71</td>
<td>12/108</td>
<td>1.79 (.77–4.19)</td>
<td>.17</td>
</tr>
<tr>
<td>HSCT donors to recipients with IA vs control donors†</td>
<td>10/68</td>
<td>17/107</td>
<td>0.91 (.39–2.13)</td>
<td>.83</td>
</tr>
<tr>
<td>Presence of Y238X in both HSCT recipient &amp; donor†</td>
<td>5/68</td>
<td>8/107</td>
<td>.98 (.27–2.80)</td>
<td>.98</td>
</tr>
</tbody>
</table>

† DNA belonging to HSCT donors of 3 IA cases and 1 control case were unavailable for genotyping. IA denotes invasive aspergillosis; HSCT: hematopoietic stem cell transplantation; OR: odds ratio; 95%CI: 95% confidence interval. P-values were obtained by Pearson-chi-square test.
Study Group criteria [11]. One hundred eight patients with comparable underlying disorders who underwent HSCT but did not develop IA were enrolled as control subjects for the HSCT recipients. All HSCT recipients with IA and the control subjects had undergone T cell–depleted allogenic HSCT. The clinical characteristics of the HSCT recipients and control subjects are summarized in Table 1.

Of the 21 non-HSCT recipients who developed IA after chemotherapy, 18 had acute myeloid leukemia; there was 1 case each of acute lymphocytic leukemia, multiple myeloma, and aplastic anemia. The median age was 50 years (interquartile range, 40–61 years), and 13 of the 21 patients (62%) were male. Seventeen of the 21 patients (81%) had probable IA, and proven IA was diagnosed in the remaining 4 patients. Prolonged neutropenia (defined as absolute neutrophil count <500 cells/mm³ for >14 days before diagnosis of IA) was present in 9 (43%) of 21 patients.

None of the patients or control subjects in this study received prior mould-active antifungal prophylaxis [12, 13]. DNA was obtained from patients after informed consent was obtained, as required by the ethical committee of each respective institution. For HSCT cases, DNA was obtained from both recipients and their respective donors before transplantation.

**Genotyping for Dectin-1 Y238X Polymorphism**

The Y238X SNP (rs16910526) in exon 6 is the only known exonic polymorphism in the Dectin-1 gene in Caucasian populations [7]. Genotyping for the presence of the Y238X polymorphism was performed using the TaqMan SNP assay C_33748481_10 on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems).

**Cytokine Stimulation Assays**

Cytokine profiling was performed to ascertain the functional consequence of the Dectin-1 Y238X polymorphism. The isolation of peripheral blood mononuclear cells (PBMCs) and differentiation of monocyte-derived macrophages (MDMs) from study subjects were performed as described elsewhere [14]. The cells were stimulated with live and heat-killed conidia and with heat-killed hyphae of a well-characterized *Aspergillus fumigatus* clinical strain, V05-27 [15]. Where indicated, *Candida albicans* blastoconidia belonging to strain ATCC MYA-3573 (UC820) [16] and particulate β-glucan (courtesy of Dr David Williams, University of Tennessee) were used as control stimuli. The supernatants were collected after 24 h of incubation at 37°C and stored at −20°C until cytokine assay. Interleukin (IL)–6 and tumor necrosis factor (TNF)–α concentrations were measured by commercial sandwich enzyme-linked immunosorbent assay kits (PeliKinectri Compact and R&D Systems, respectively) according to the manufacturers’ instructions.

**Flow Cytometry**

Freshly isolated human PBMCs were incubated with 5 μg/mL murine anti-Dectin-1 mAb 259931 (R&D Systems) or mouse IgG2b isotype control in RPMI 1640 (supplemented with 2% human serum), followed by allophycocyanin-conjugated goat anti-mouse antibody (BD Pharmingen). Monocytes were labeled with anti–CD14-PE (Pelicluster), and Dectin-1 expression on CD14⁺ cells was determined using flow cytometry (FACS Scilibur; BD Biosciences). Detection of Dectin-1 receptor surface expression on MDM was performed as described above. Surface mannose receptor (MR) and TLR2 and TLR4 expression was determined using anti–MR-FITC (R&D Systems), anti–TLR2-FITC, and anti–TLR4-PE (both from eBioscience), in addition to their respective isotype controls.

**Statistical Analysis**

Genotype frequencies were compared between groups with use of Fisher’s exact and Pearson χ² tests. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated for the presence (homozygous or heterozygous) or absence (homozygous wild-type [WT] allele) of the Dectin-1 Y238X polymorphism. Multivariate adjustments for neutropenia and development of graft-versus-host disease were made where appropriate. The influence of the variant Dectin-1 SNP on the clinical course of disease (ie, day from HSCT or start of chemotherapy to the day of diagnosis) was assessed using Kaplan–Meier analysis (log rank test). Likewise, associations with presence of the polymorphism and time from IA diagnosis to death were assessed. The cytokine data were presented as mean plus standard error of the mean. Differences in cytokine production were assessed using Student’s t test. A P value <.05 was considered to be statistically significant. SPSS, version 17.0 (SPSS for Windows), was used to perform the calculations.

**Table 2b. Comparison of allele frequencies of Dectin-1 Y238X variant in susceptible patient cohorts and healthy populations.**

<table>
<thead>
<tr>
<th>Study Cohort</th>
<th>Non-HSCT patients with IA</th>
<th>HSCT patients (with- and without IA)</th>
<th>Healthy HSCT donors</th>
<th>Correspoding Healthy Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dectin-1 Y238X variant</td>
<td>7/21</td>
<td>25/179</td>
<td>27/175</td>
<td>19/138</td>
</tr>
<tr>
<td>Allele frequency</td>
<td>19.0%</td>
<td>7.0%</td>
<td>7.7%</td>
<td>6.9%</td>
</tr>
</tbody>
</table>

**NOTE.** IA: invasive aspergillosis. †: All individuals were heterozygous for the Y238X Dectin-1 polymorphism except for one individual in the Non-HSCT group who was homozygous. #: Dutch healthy population [6]. x: Frequency of the allele frequency was significantly higher as compared to the all three other populations (Fishers exact test: P < .05), see text for details.
RESULTS

Dectin-1 Y238X Polymorphism in Patients with IA and Control Subjects

After HSCT, the primary immune cells of the recipient will eventually assume genotype of the donor after successful engraftment. Thus, presence of the Dectin-1 Y238X SNP was determined in all patients and HSCT donors. The genotype frequencies of the study cohort were in Hardy Weinberg equilibrium. Thirteen of the 71 patients (18.3%) who developed IA after HSCT and 12 (11.1%) of 108 control patients had the Dectin-1 Y238X SNP. All these individuals were heterozygous for the SNP. Possession of the Y238X polymorphism was only associated with a limited trend towards IA susceptibility, and this did not reach statistical significance (OR, 1.79; 95% CI, .77–4.19; P = .17) (Table 2a). After multivariate adjustment for neutropenia and graft-versus-host disease, the adjusted OR was 1.70 (95% CI, .72–4.00; P = .22). Donor genotype did not influence risk of IA in the recipient. Likewise, simultaneous possession of Y238X in both HSCT donor-recipient pair did not increase susceptibility to IA.

In addition, the Dectin-1 Y238X SNP was found in 7 (33.3%) of the 21 non-HSCT patients who developed IA after immunosuppressive chemotherapy; 1 individual was homozygous for the Y238X polymorphism. Because of the limited case patients in this non-HSCT cohort, we opted to compare the allelic frequencies of Dectin-1 Y238X variant against the following patient cohorts and healthy populations: (1) the HSCT recipients with and without IA in this study who had similar underlying hematological diseases, (2) the healthy HSCT donors (in this study), and (3) healthy population of comparable Dutch ancestry [6] (Table 2b). The allele frequency of the Y238X SNP was significantly elevated in the non-HSCT patients with IA (19.0%), compared with HSCT recipients (7.0%; P = .01), healthy HSCT donors (7.7%; P = .04), and the Dutch population (6.9%; P = .02).

Influence of Dectin-1 Polymorphism on Clinical Course of IA

In addition to its effect on the susceptibility to IA, we assessed whether the presence of the Dectin-1 variant gene might influence clinical course during IA. Kaplan-Meier analysis did not reveal a difference in time to development of IA from HSCT between recipients or their donors bearing either the WT or variant allele (P = .94 and P = .88, respectively) (Figure 1a and b). There was no difference in survival (time to death after diagnosis of IA) consequent to having the WT or variant Dectin-1 allele in both patients and donors (P = .83 and P = .99, respectively, by log rank) (Figure 2a and 2b).

Functional Consequences of the Dectin-1 Y238X Polymorphism

Functional assays were performed to find a mechanistic explanation on the limited influence of Dectin-1 on susceptibility to IA. To fully elucidate the phenotypic effects of the Dectin-1 Y238X polymorphism, we used Dectin-1-deficient PBMCs and differentiated MDMs from 2 siblings whom we characterized as homozygous for the variant Dectin-1 allele [6] and from healthy control subjects who had WT Dectin-1 gene.

Cytokine Stimulation

We assessed the capacity of the immune cells to respond to the various stimuli. In PBMCs, homozygosity for Dectin-1 Y238X resulted in marked reduction of proinflammatory TNF-α and IL-6 production in response to heat-killed A. fumigatus hyphae, C. albicans blastoconidia, and live A. fumigatus conidia, as would have been anticipated because of the key role that the Dectin-1 receptor is known to play in recognition of fungal cell wall β-glucan (Figure 3a and b) [7, 9]. In the MDM, however, there

Figure 1. Kaplan-Meier plot on time (days) to diagnosis of IA after HSCT conditioning or chemotherapy in patients and HSCT donors who have either the Y238X polymorphism (Dectin-1 variant) or wild-type Dectin-1.
were no significant differences in proinflammatory cytokine responses between subjects who were homozygous or WT for Dectin-1. This was apparent for both live *A. fumigatus* conidia and heat-killed hyphae (Figure 3c and d). As control, stimulation using β-glucan still failed to generate TNF-α response in the Dectin-1–deficient MDMs, in contrast to WT Dectin-1 MDMs. Despite the intrinsic inability to signal via the Dectin-1 pathway in the Dectin-1-deficient MDMs, the demonstrated ability of these cells to still respond to *Aspergillus* indicated the presence of alternative signaling pathways.

**Flow Cytometry**

We had previously revealed that monocytes from individuals homozygous for Y238X polymorphism had diminished Dectin-1 receptor cell surface expression [7]. In the present study, we further revealed that MDMs from these individuals also had deficient expression of the Dectin-1 receptor (Figure 4a and c); this finding is is corroborated by the aforementioned inability to respond to β-glucan. In addition to Dectin-1, other PRRs, such as the mannose receptor, TLR2, and TLR4, participate in recognition of fungal ligands [5]. Of note, the mannose receptor is a distinct C-type lectin receptor frequently found mainly on macrophages, and TLR2 and TLR4 are ubiquitous on most immune cells, including monocytes and macrophages. It is plausible that the host tissue macrophages recognize *Aspergillus* through these alternative PRRs, especially the mannose receptor pathway. This may circumvent deficiency in the Dectin-1 signaling pathway and account for the normal cytokine production in Dectin-1–deficient MDMs. To substantiate this, we showed that Dectin-1–deficient MDMs had similar levels of MR expression as those in normal cells (Figure 4b and d) and that TLR2 and TLR4 expression was normal (data not shown).

**DISCUSSION**

Although much has been reported on the central role that Dectin-1 plays in host recognition of *Aspergillus*, this is based largely on findings from experimental murine models. In the present study, however, we found that a defective function of Dectin-1 resulting from a premature stop codon polymorphism may potentially enhance susceptibility to IA in susceptible non-HSCT patients, although the effect was moderate in the HSCT cohort and did not significantly alter the clinical course of the disease. In contrast to the observations on Dectin-1 obtained from in vitro and mice models, the aforementioned clinical findings remain significant, because they also highlight the system of redundancy inherent in the human innate immune system against invading pathogens such as *Aspergillus*.

Recently, Dectin-1 was recognized as being a pivotal PRR for the control of fungal infections [17] and, specifically, for anti-*Aspergillus* host defense [10]. In vitro studies revealed the involvement of Dectin-1 in both TLR-dependent and TLR-independent antifungal responses [14, 18]. The clinical significance of Dectin-1 in mucosal candidiasis was highlighted recently in a study that described how defective Dectin-1 expression and function resulted in recurrent vulvovaginal candidiasis in a family of siblings who were homozygous for the Y238X polymorphism [6], and another study reported an increased incidence of oral and gastrointestinal Candida colonization in HSCT recipients heterozygous for the same variant gene [7].

Despite the in vitro studies pointing out the importance of Dectin-1 as a receptor for fungal β-glucans, the perceived importance of Dectin-1 for invasive mycosis in mice models had been debated. Contrary to findings of Taylor et al [19] that Dectin-1–deficient mice showed increased susceptibility to disseminated candidiasis, another study by Saijo et al [20] did not
yield similar corresponding results with use of an independently developed Dectin-1−/− mice strain. A later study showed that Dectin-1 had an important role in a murine model of IA [10]. Nevertheless, a family with siblings who had a complete deficiency of the Dectin-1 function did not report susceptibility to systemic fungal infections [6]. This suggests that, although Dectin-1 has an unchallenged role as β-glucan receptor, in the in vivo situation, alternative recognition pathways can initiate effective antifungal responses.

Patient studies remain crucial for the validation of the host defense mechanisms identified in in vitro and experimental studies. To be at risk for development of IA, a profoundly immunocompromised status consequent to immune-ablative chemotherapy, HSCT conditioning regimens, or chronic corticosteroid treatment is obligate. In the present study, we found a markedly increased Y238X allele frequency of 19.0% in non-HSCT patients who developed IA after chemotherapy, compared with other reference populations (range, 6.9%–7.7%). In concordance with findings from a previous study that reported the Y238X allele frequency to be 6.9% in the general Dutch population, our analysis of healthy donors to HSCT recipients also yielded a comparable allele frequency of 7.7% (P = .76). One consideration would have been whether the possession of the Y238X polymorphism could remotely be related to acquisition and progression of the underlying hematological disease state, resulting in an over-represented allele frequency in the non-HSCT patients with IA. However, this was not the case, because we also determined that, in the HSCT recipients with similar predisposing hematological disorders, the allele frequency of Y238X was 7.0%; this was comparable to that in the aforementioned healthy populations.

The occurrence of IA in HSCT presents a challenge in studying genetic susceptibility, because both donor and recipient genotype will invariably exert their influence on function of the immune cells after transplantation. Even after documented engraftment, it remains unresolved when chimerism is actually achieved at the level of the pulmonary macrophages that form the front line against the invading Aspergillus. We found that Y238X status in HSCT recipients was associated with a modest trend toward susceptibility to IA (OR, 1.79; 95% CI, .77–4.19;
This was not accentuated after multivariate adjustment (adjusted OR, 1.70; 95% CI, 0.72–4.00; \( P = .22 \)). In addition, donor Y238X status was found not to be an attributory factor. Simultaneous presence of the Dectin-1 Y238X variant in both donor-recipient pair did not further confer a dose-dependent effect on susceptibility to IA (Table 2a). Immune recognition and activation at the epithelial level is a key mechanism in host defense against invasive pathogens [21]. After HSCT, Dectin-1 expression on epithelial cells and pneumocytes remains as determined by recipient genotype in contrast to immune cells of myeloid origin. Thus, this reasonably accounts for our finding that it was the recipient Dectin-1 Y238X status, rather than the donor, that had an influence on susceptibility to IA.

The stronger association observed in the non-HSCT cohort despite the smaller patient numbers may be attributable to patient and treatment profiles being relatively more homogenous. Comparisons incorporating both cohorts, however, may be confounded, because there remain inherent differences in treatment regimens (and possibly IA susceptibility) between non-HSCT and HSCT patients [22]. Nevertheless, the increased incidence of the Dectin-1 Y238X variant in non-HSCT patients with IA and its association towards IA susceptibility in HSCT recipients suggest that heterozygosity for the Y238X SNP has a moderate association with acquisition of IA in at-risk patients. Recognizing the potential limitation of our finding, pertaining specifically to the smaller non-HSCT study cohort and to the methodology used for the subgroup analysis, further validation of this observation in a larger cohort of non-HSCT patients with IA and control subjects is needed.

Our functional assays using cells isolated from individuals homozygous for the Dectin-1 Y238X polymorphism also shed light on why susceptibility to Aspergillus infection may be limited and clinical course of disease relatively unaltered despite reduced Dectin-1 receptor function. In contrast to the PBMCs, Dectin-1–defective MDMs had the capability to respond with normal production of proinflammatory cytokines after challenge with \( A. fumigatus \). Because pulmonary macrophages form the first line of defense against inhaled \( A. fumigatus \) conidia, our findings highlight that the capacity of macrophages to retain their response even with deficient Dectin-1 function probably lies in their capacity to engage alternative PRRs: MR, TLR2, and TLR4. These receptors are known to be involved in immune recognition of \( A. fumigatus \) and antifungal host defense [23–26]. This ability to retain the capacity to respond to the pathogen in the absence of Dectin-1 underscores the redundancy that is inherent to the human antifungal host defense. On the other hand, the modest susceptibility to aspergillosis in patients bearing the Y238X polymorphism, coupled with the defective monocyte function, suggest an adjuvant yet essential role of infiltrating monocytes for host defense. In contrast to monocytes and macrophages, the main \( \beta \)-glucan receptor on neutrophils is complement receptor 3 [27]. Although neutrophils are important in anti-\( A. fumigatus \) host defense [28], the Dectin-1 Y238X polymorphism does not affect these cells, because neutrophil function was normal in individuals with homozygous Dectin-1 Y238X mutation [6].

Other polymorphisms in genes coding for components of the innate immunity have been recently reported to increase susceptibility to Aspergillus infections: TLR1, TLR4, TLR6, and IL-10 promoter [29–34]. In the aforementioned studies, similar to ours, the polymorphism of interest was studied in isolation and not in association with each other. It is tempting to consider that the concomitant presence of \( \geq 2 \) of these polymorphisms in a patient may further enhance the risk profile to IA.
In conclusion, in the present study, we report that the Dectin-1 Y238X polymorphism was associated with a moderate increase in susceptibility to IA, particularly in non-HSCT immunocompromised patients. Additional studies are needed to validate these findings, but these data provide novel insight in human host defense during IA.

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References