Role of the Mycobiome in Human Acute Graft-versus-Host Disease

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A role for gut bacteria in the pathogenesis of graft-versus-host disease (GVHD) has been firmly established; however, the role of *Candida* spp, which form part of the mycobiome, remains unknown. In a homogenous group of patients who underwent allogeneic stem cell transplantation (SCT), we found a significant impact of *Candida* colonization on the occurrence of acute GVHD. Patients colonized with *Candida* spp developed significantly more grade II-IV acute GVHD compared with noncolonized patients (50% vs 32%; $P = .03$), as well as more gastrointestinal (GI)-GVHD (33% vs 19%; $P = .05$). Colonization with *Candida* spp was more frequent in patients bearing the loss-of-function polymorphism Y238X, which results in dectin-1 dysfunction, compared with patients with the wild-type allele (73% vs 31%; $P = .002$). There was no direct effect of dectin-1 dysfunction on acute GVHD, although it did influence the occurrence of GVHD indirectly through *Candida* colonization. The exact mechanism of GVHD induction by *Candida* spp colonization of the mucosa is unknown, but the link might prove to be the induction of Th 17/IL-23 responses through activation of pattern recognition receptors by fungal motifs, including β-D-glucan and mannans. These data indicate a role for the mycobiome in the pathogenesis of GVHD and suggest that altering the mycobiome by antifungal drugs can help ameliorate GI-GVHD. In addition, given that the genetic constitution of patients affects susceptibility to both *Candida* colonization and GVHD, whether identifying gene polymorphisms will facilitate personalized treatment of SCT recipients remains to be determined.

INTRODUCTION

Recently, interest has been revived in the role of mucosal innate immunity and the gastrointestinal (GI) microbiota in the pathogenesis of both chemotherapy-induced intestinal mucositis and graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (SCT) [1,2]. The interactions among the innate immune system, gut commensal bacteria, and donor T cells are complex and often multidirectional. On the one hand, perturbed mucosal innate immunity resulting from cytotoxic therapy or gene polymorphisms, along with changes in the composition and number of gastrointestinal microbiota (dysbiosis), can aggravate inflammation of the intestinal mucosal barriers and stimulate alloreactive T cell responses, inducing GVHD [3,4]. On the other hand, damage to the GI tract induced by GVHD alters the host response by, for instance, T cell–mediated Paneth cell damage, resulting in decreased release of α-defensin, with subsequent dysbiosis and increased risk for bacteremia, fungemia, and perpetuation of GVHD [5].

A role for gut bacteria in the pathogenesis of GVHD was postulated almost 40 years ago [6], and selective gut decontamination has been associated with a decreased incidence of acute GVHD [7]. However, the role of intestinal *Candida* spp, which forms part of the mycobiome, has not yet been studied in GVHD. Given the similarities between inflammatory bowel disease (IBD) and GI-GVHD, understanding the role of *Candida* spp in IBD might provide insight into a possible role in GI-GVHD as well [8]. Iliev et al. [9] recently reported an important role of the mycobiome and changes therein, in the severity of experimental colitis in animals. Defective innate immune responses caused by dectin-1 deficiency resulted in increased colonization with *Candida* spp, which aggravated chemically induced colitis; these effects were counteracted by treatment with fluconazole. Dectin-1 is a pivotal pattern recognition receptor that recognizes the β-D-glucan found in fungal cell walls, including those of *Candida* and triggers mucosal antifungal $CD4^+$ Th17 responses [10]. The release of IL-17 and IL-22 is a key aspect of mucosal antifungal defenses, stimulating the release of antimicrobial peptides, including Reg proteins and defensins and recruitment of neutrophils. Interestingly, α- and β-defensins also have strong antimicrobial activity against *Candida* species. Dysfunctional dectin-1 has clinical implications for humans, with the loss-of-function single-nucleotide polymorphism (SNP; Y238X, rs16910526) linked to increased susceptibility to mucosal fungal infections [10].

Given the relationship between *Candida* colonization and IBD, we wondered whether the intestinal mycobiome also might play a role in the pathogenesis of GVHD, particularly in GI-GVHD. Thus, we investigated whether *Candida* colonization and dectin-1 function had any effect on the development of GVHD.

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Table 1
Patient, Donor, and Transplantation Characteristics

Characteristic	Value
Patient age, yr, median (range)	48 (18-64)
Patient sex, male/female, n	97/56
Donor age, yr, median (range)	46 (20-65)
Donor sex, male/female, n	92/61
Donor–recipient sex match, n (%)	
Female donor–male recipient	41 (27)
Other	112 (73)
Diagnosis, n (%)	
AML/MDS/ALL	102 (67)
Other	51 (33)
Myeloablative conditioning, n (%)	
Idarubicin-cyclophosphamide-TBI	103 (67)
Idarubicin-cyclophosphamide-busulfan	13 (9)
Cyclophosphamide-TBI	29 (19)
Cyclophosphamide-busulfan	8 (5)
T cell depletion technique, n (%)	
Elutriation	33 (21.5)
CD34 selection	65 (42.5)
CD3/CD19 depletion	55 (36)
Stem cell source, n (%)	
Bone marrow	48 (31)
Peripheral blood	105 (69)
Graft $CD34^+$ cells, $\times 10^6$/kg, median, range	3.0 (0.8-11.0)
Graft $CD3^+$ cells, $\times 10^6$/kg, median, range	0.5 (0.3-0.7)
GVHD prophylaxis with cyclosporine A, n (%)	153 (100)
Acute GVHD, n (%)	
Grade II-IV	59 (38.5)
Grade III-IV	25 (16)
Acute GI-GVHD grade I-IV, n (%)	37 (24)
Candida colonization, n (%)	54 (35)

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; TBI, total body irradiation.

PATIENTS AND METHODS

Study Population and Treatment Protocol

We performed a retrospective analysis in a highly homogenous group of 153 patients who had undergone matched-related partially T cell–depleted allogeneic SCT after myeloablative conditioning (Table 1). All patients had received only cyclosporine for GVHD prophylaxis, along with selective antimicrobial prophylaxis consisting of ciprofloxacin during the first weeks after SCT [11].

Candida colonization was evaluated within the first 7 to 10 days after admission and was defined as the presence of *Candida* spp in a fecal sample and mouthwash sample obtained on the same day, or recovery of *Candida* spp from samples obtained from the same site on 2 consecutive occasions. Fluconazole 200 mg/day was prescribed only for those patients colonized with *Candida albicans*, *Candida tropicalis*, or *Candida parapsilosis* and not for patients colonized with species likely to be resistant to the drug, such as *Candida krusei* and *Candida glabrata*. Acute GVHD and GI-GVHD were scored according to the criteria of Przepiorka et al. [12].

Detection of Dectin-1 SNP Y238X

DNA for genotyping was available for a subset of 127 patients and their donors. Genotyping for the presence of the Y238X polymorphism in the patient group was performed as described previously [11]. All patients provided informed consent for the prospective collection of DNA samples for investigational use.

Statistical Analysis

We first performed a univariate analysis of the impact of *Candida* colonization and other factors on the development of acute GVHD grade II-IV and GI-GVHD grade I-IV. Factors with $P < .20$ in the univariate analysis were then incorporated into a backward logistic regression analysis; the criterion for remaining in the model was a likelihood ratio associated with $P < .05$. The chi-square test was used to compare the rate of *Candida* colonization in patients with and without the dectin-1 polymorphism. SPSS software (IBM, Armonk, NY) was used for statistical analyses and generation of probability curves for GVHD. A P value $< .05$ was considered statistically significant.

RESULTS

Candida Colonization Increases GVHD

The overall incidence of acute GVHD and GI-GVHD after partially T cell–depleted SCT with myeloablative conditioning was 38.5% (59 of 153) and 24% (37 of 153), respectively. Approximately 35% of the patients (54 of 153) were colonized, mainly with *C albicans* (85%), with *C glabrata*, *C krusei*, *C parapsilosis*, and *C tropicalis* accounting for the remainder. Patients colonized with *Candida* spp had a significantly higher rate of grade II-IV acute GVHD compared with uncolonized patients (50% [27 of 54] vs 32% [32 of 99]; OR, 2.0; 95% confidence interval [CI], 1.05-4.13; $P = .038$) (Table 2 and Figure 1A). After multivariate analysis, *Candida* colonization was the only significant factor remaining ($P = .034$), with stem cell source and method of T cell depletion no longer significant. Patient colonized with *Candida* spp were also more prone to developing GI-GVHD, but the difference failed to reach nominal statistical significance (33% [18 of 54] vs 19% [19/99]; OR, 2.11; 95% CI, 0.99-4.48; $P = .07$) (Table 2 and Figure 1B). Backward logistic regression analysis, however, showed significant relationships between GI-GVHD and *Candida* colonization ($P = .049$) and stem cell source ($P = .016$) (Table 2). In our cohort, 45 patients experienced isolated grade I-IV skin GVHD. Colonization with *Candida* spp had no significant impact on the incidence of isolated skin GVHD (48% [15 of 31] of patients colonized with *Candida* vs 42% [30 of 71] of noncolonized patients; $P = .66$).

Fluconazole 200 mg/day was prescribed in 83% (45 of 54) of the patients colonized with *Candida* spp. The patients not receiving fluconazole were colonized mainly with *C glabrata* and *C krusei*. Fluconazole therapy was initiated late, in the second or third week after admission, in patients with established neutropenia and mucosal barrier damage. Thus, although the fungal burden was decreased in some patients, eradication of *Candida* spp was achieved in only 35% (16 of 45) of colonized patients. Colonized patients who received fluconazole had a slightly lower risk of developing GVHD compared with colonized patients who did not receive fluconazole (47% [21 of 45] vs 67% [6 of 9]); however, the number of patients was too small to allow detection of a significant difference ($P = .47$).

Dectin-1 Polymorphism Y238X, Candida Colonization, and GVHD

Fifteen patients (12%) were heterozygous for the dectin-1 SNP, and 112 (88%) were homozygous for the wild-type allele. The genotype frequencies in the study cohort showed Hardy-Weinberg equilibrium with an allele frequency of 6%, as has been reported previously. Colonization with *Candida* spp was more frequent in patients bearing the dectin-1 SNP compared with those with the wild-type allele (73% [11 of 15] vs 31% [35 of 112]; $P = .002$). Dectin-1 mutation status had no impact on the incidence of acute GVHD, being 30% in both groups (Figure 2); nevertheless, 45% (5 of 11) of the patients with both the dectin-1 mutation and *Candida* colonization had acute GVHD, a rate similar to that in the total group of colonized patients. Conversely, none of the 4 noncolonized patients with the mutation had acute GVHD; however, the number of patients is too small to permit a meaningful statistical analysis.

DISCUSSION

We found a significant impact of *Candida* colonization on the rates of acute GVHD, and of GI-GVHD, in a homogenous group of patients who underwent allogeneic SCT. However, no

Table 2
Univariate and multivariate analysis of risk factors for acute GVHD and GI-GVHD

Variable	No.	Acute GVHD grade II-IV			Acute GI-GVHD		
		OR (95% CI)	*P* Value*	*P* Value†	OR (95% CI)	*P* Value*	*P* Value†
Conditioning							
No total body irradiation	21	1			1		
Total body irradiation	132	1.68 (0.61-4.60)	.35	—	2.08 (0.58-7.51)	.41	—
Diagnosis							
Other diagnoses	51	1			1		
AML/MDS/ALL	102	1.59 (0.78-3.24)	.22	—	1.48 (0.65-3.35)	.43	—
Donor–recipient sex match							
Other	112	1			1		
Female donor–male recipient	41	0.89 (0.43-1.87)	.89	—	1.21 (0.54-2.75)	.67	—
T cell depletion							
Elutriation	33	1			1		
CD34 selection	65	1.35 (0.55-3.3)	.65		3.00 (0.80-11.23)	.11	
CD3/CD19 depletion	55	1.92 (0.77-4.77)	.18	NS	5.28 (1.42-19.57)	.01	NS
Age group							
<50 yr	88	1			1		
≥50 yr	65	1.24 (0.64-2.40)	.6	—	1.61 (0.76-3.38)	.25	—
Stem cell source							
Bone marrow	48	1			1		
Peripheral blood	105	1.82 (0.86-3.79)	.11	NS	2.93 (1.13-7.60)	.025	.016
Candida colonization							
No	99	1			1		
Yes	54	2.09 (1.05-4.13)	.038	.033	2.11 (0.99-4.48)	.07	.049
Dectin-1 status‡							
Wild-type	112	1			1		
Polymorphism	15	1.06 (0.34-3.34)	1.00	—	1.22 (0.31-4.78)	.72	—

NS indicates not significant; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome.
* Univariate analysis (chi-square test).
† Backward logistic regression analysis; only factors with a *P* value < .20 on univariate analysis were incorporated.
‡ Dectin-1 status was determined in 127 patients.

impact of *Candida* colonization was seen in patients with isolated acute skin GVHD. There was also a strong association between dectin-1 dysfunction, caused by the loss-of-function SNP Y238X and mucosal *Candida* colonization in this setting, as reported previously [11]. Although the subgroup of patients carrying the dectin-1 mutation was small, there seems to be no direct effect of dectin-1 dysfunction on acute GVHD, suggesting that dectin-1 dysfunction influences GVHD only indirectly through *Candida* colonization. Our human data are in line with findings in an IBD mouse model reported by Iliev et al. [9]. Moreover, our findings are supported by the results of Marr et al. in 2000 [13]. Although designed to assess the impact of fluconazole prophylaxis (400 mg/day starting at the initiation of conditioning therapy and extending for up to 120 days) on *Candida* infection and candidiasis-related mortality in allogeneic SCT, that study also found a decreased incidence of GI-GVHD in the patients receiving fluconazole compared with those who did not receive fluconozole (5.5% vs 14%; $P = .02$). In our cohort, the use of fluconazole was not associated with a significant reduction in GVHD; however, but this lack of effect was likely related to the delayed start and lower dose of fluconazole therapy, resulting in a late and modest effect on fungal burden in colonized patients, demonstrated by an eradication rate of only 35%.

The exact mechanism of GVHD induction by *Candida* spp colonization of the mucosa is currently unknown, but there likely are similarities with the mechanisms through which gut bacteria aggravate GVHD [1]. Translocation of *Candida* motifs, such as β-D-glucan and mannans, during conditioning therapy–induced mucosal damage can aggravate both local mucosal and systemic immune responses through activation of different pattern recognition receptors (PRRs), most

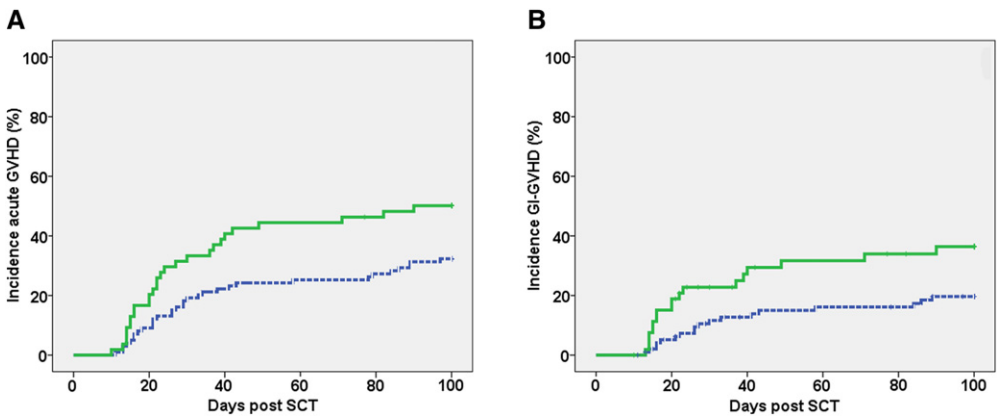

Figure 1. Incidence of acute GVHD in relation to *Candida* colonization. Shown are time-dependent occurrence of GVHD grade II-IV (A) and GI-GVHD (B) in patients colonized with *Candida* spp (green line) and patients not colonized (blue line).

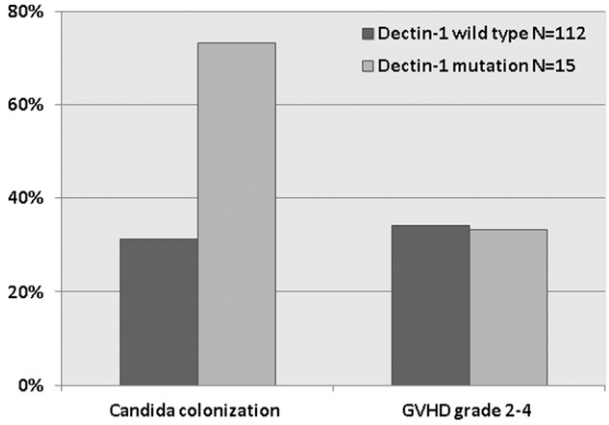

Figure 2. *Candida* colonization, dectin-1 status, and GVHD. Dectin-1 single-nucleotide polymorphism Y238X resulted in significantly more *Candida* colonization but had no direct effect on acute GVHD grade II-IV.

importantly dectin-1, dectin-2, mannose receptor, and Toll-like receptor 2 [14]. These PRRs are expressed on several types of immune cells, including dendritic cells and other antigen-presenting cells and cause the release of proinflammatory cytokines and induce Th1 and Th17 responses on activation. Th17 responses are known to be most important in anti-*Candida* host defenses at the mucosal barrier [15,16], and interestingly, recent studies have also revealed a role for Th17/IL-23 in the pathogenesis of acute GVHD in both animals and humans, including GI-GVHD [17-19]. Thus, the link between *Candida* colonization and acute GVHD might prove to be in the induction of Th17/IL-23 responses, especially when the fungus is present in the gut. However, dectin-1 dysfunction owing to the Y238X polymorphism results in decreased mucosal Th17 responses [20], which on the one hand explains the increased *Candida* colonization, but on the other hand also suggests that dectin-1 dysfunction might ameliorate GVHD. This seems to not be the case, however, as demonstrated by our data. The most likely explanation is the existence of some degree of redundancy in PRR activation by *Candida* spp. Thus, despite the dectin-1 dysfunction, the activation of other PRRs by the presence of an increased fungal burden increases mucosal immune responses that aggravate GVHD. Clearly, additional experimental studies are needed to shed light on these hypotheses.

In conclusion, colonization with *Candida* has a significant influence on the occurrence of acute GVHD, including GI-GVHD, in humans, indicating a role of the mycobiome in the pathogenesis of GVHD. The dectin-1 SNP Y238X mutation increases the likelihood of *Candida* colonization but appears to have only an indirect role in causing GVHD. A role of gut bacteria has long been implicated in the pathogenesis of acute GVHD, and although the focus to now has been on gut bacteria, our data reported here underscore the importance of the gut mycobiome. The use of fluconazole and other antifungal drugs in SCT recipients is being increasingly advocated to prevent fungal infections, but our findings suggest that it might ameliorate GI-GVHD as well. However, timely initiation of antifungal drugs (ie, at the start of the conditioning) at the proper dosage might prove essential for effective reduction of the fungal burden and thus protection against GVHD. It would be of interest to test whether altering the mycobiome composition by means other than antifungal drugs, such as with the use of prebiotics or probiotics, can be exploited to reduce the incidence of GVHD. Given that an individual's genetic constitution affects susceptibility to both *Candida* colonization and GVHD, it remains to be seen whether identifying the gene polymorphisms will help achieve personalized treatment of SCT recipients, exposing only those who will benefit from antifungal agents, thereby reducing the use of drugs and any concomitant side effects, decreasing the risk of selecting antifungal resistance, and helping control associated costs.

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REFERENCES

1. Penack O, Holler E, van den Brink MR. Graft-versus-host disease: regulation by microbe-associated molecules and innate immune receptors. *Blood*. 2010;115:1865-1872.
2. van Vliet MJ, Harmsen HJ, de Bont ES, et al. The role of intestinal microbiota in the development and severity of chemotherapy-induced mucositis. *PLoS Pathog*. 2010;6:e1000879.
3. Penack O, Smith OM, Cunningham-Bussel A, et al. NOD2 regulates hematopoietic cell function during graft-versus-host disease. *J Exp Med*. 2009;206:2101-2110.
4. Jenq RR, Ubeda C, Taur Y, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. *J Exp Med*. 2012;209:903-911.
5. Eriguchi Y, Takashima S, Oka H, et al. Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of alpha-defensins. *Blood*. 2012;120:223-231.
6. van Bekkum DW, Roodenburg J, Heidt PJ, et al. Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. *J Natl Cancer Inst*. 1974;52:401-404.
7. Vossen JM, Heidt PJ, van den Berg H, et al. Prevention of infection and graft-versus-host disease by suppression of intestinal microflora in children treated with allogeneic bone marrow transplantation. *Eur J Clin Microbiol Infect Dis*. 1990;9:14-23.
8. Pineton de Chambrun G, Colombel JF, Poulain D, et al. Pathogenic agents in inflammatory bowel diseases. *Curr Opin Gastroenterol*. 2008; 24:440-447.
9. Iliev ID, Funari VA, Taylor KD, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science*. 2012;336:1314-1317.
10. Ferwerda B, Ferwerda G, Plantinga TS, et al. Human dectin-1 deficiency and mucocutaneous fungal infections. *N Engl J Med*. 2009;361:1760-1767.
11. Plantinga TS, van der Velden WJ, Ferwerda B, et al. Early stop polymorphism in human *DECTIN-1* is associated with increased *Candida* colonization in hematopoietic stem cell transplant recipients. *Clin Infect Dis*. 2009;49:724-732.
12. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15: 825-828.
13. Marr KA, Seidel K, Slavin MA, et al. Prolonged fluconazole prophylaxis is associated with persistent protection against candidiasis-related death in allogeneic marrow transplant recipients: long-term follow-up of a randomized, placebo-controlled trial. *Blood*. 2000;96:2055-2061.
14. van de Veerdonk FL, Kullberg BJ, Van der Meer JW, et al. Host–microbe interactions: innate pattern recognition of fungal pathogens. *Curr Opin Microbiol*. 2008;11:305-312.
15. Conti HR, Shen F, Nayyar N, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med*. 2009;206:299-311.
16. Eyerich K, Foerster S, Rombold S, et al. Patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. *J Invest Dermatol*. 2008;128: 2640-2645.
17. Das R, Chen X, Komorowski R, et al. Interleukin-23 secretion by donor antigen-presenting cells is critical for organ-specific pathology in graft-versus-host disease. *Blood*. 2009;113:2352-2362.
18. Bossard C, Malard F, Arbez J, et al. Plasmacytoid dendritic cells and Th17 immune response contribution in gastrointestinal acute graft-versus-host disease. *Leukemia*. 2012;26:1471-1474.
19. Dander E, Balduzzi A, Zappa G, et al. Interleukin-17–producing T-helper cells as new potential player mediating graft-versus-host disease in patients undergoing allogeneic stem cell transplantation. *Transplantation*. 2009;88:1261-1272.
20. van de Veerdonk FL, Marijnissen RJ, Kullberg BJ, et al. The macrophage mannose receptor induces IL-17 in response to Candida albicans. *Cell Host Microbe*. 2009;5:329-340.