ASBMT American Society for Blood and Marrow Transplantation

Genetic Variations of Interleukin-23R (1143A>G) and BPI (A645G), but Not of NOD2, Are Associated with Acute Graft-versus-Host Disease after Allogeneic Transplantation

Martin Wermke,[1] *Stefanie Maiwald,*[2] *Renate Schmelz,*[1] *Christian Thiede,*[1] *Johannes Schetelig,*[1] *Gerhard Ehninger,*[1] *Martin Bornhäuser,*[1] *Ralf Wassmuth*[2]

Single nucleotide polymorphisms (SNPs) in genes of the immune system predict for aGVHD and mortality after allo-SCT. We investigated the effect of SNPs in the *NOD2, BPI,* and *IL-23R* genes on posttransplantation outcome in a cohort of 304 patients. *NOD2* patient and donor genotype and *BPI* recipient genotype were not associated with the occurrence of aGVHD. However, *IL-23R*-SNP in the donor was correlated with less aGVHD. This association could be confirmed in multivariate analysis (odds ratio [OR], 0.39; $P = .039$), which identified in vivo T cell depletion (OR, 0.32; $P < .001$) and multiagent GVHD prophylaxis (OR, 0.51; $P = .031$) as other independent factors predicting for less-severe aGVHD. This multivariate model also revealed a trend toward less aGVHD in patients receiving a *BPI* G allele transplant (OR, 0.60; $P = .067$) and in those receiving a transplant from an HLA-matched donor (OR, 0.57; $P = .058$). In contrast, relapse was more frequent in patients with *NOD2*-SNPs (46.2% for SNP vs 33.2% for wild-type; $P = .020$). This association was found to be of borderline significance in multivariate analysis. Neither *BPI* nor *IL-23R* genotype predicted for relapse, and none of the investigated SNPs was correlated with 5-year overall survival. In our analysis, *NOD2* SNPs did not predict aGVHD, but *IL-23R*(1142A>G) and *BPI*(A645G) SNPs appeared to be promising markers in this regard. The importance of these markers in prediction models for GVHD and relapse remain to be defined in large prospective clinical trials.

Biol Blood Marrow Transplant 16: 1718-1727 (2010) © *2010 American Society for Blood and Marrow Transplantation*

KEY WORDS: Single nucleotide polymorphism, CARD15, Innate immunity, IL-23R, Bactericidal permeability-increasing protein

INTRODUCTION

Allogeneic stem cell transplantation (allo-SCT) represents the only treatment offering cure, or at least long-term progression-free survival, for many leukemia and lymphoma patients. Despite recent advances, however, treatment-related mortality (TRM) remains at disappointingly high levels, with acute and chronic graft-versus-host disease (GVHD) being one of the main causes of treatment failure. Efforts to separate these potentially lethal complications from beneficial graft-versus-tumor effects have not been widely successful so far. Thus, scientific interest has shifted somewhat from improving HLA matching to understanding the contribution of minor histocompatibility antigens and the innate immune system (reviewed by Penack et al. [1]). A milestone in the latter area was the identification of single nucleotide polymorphisms (SNPs) in the *NOD2/CARD15* gene as predictors for the occurrence of acute GVHD (aGVHD) by Holler et al. [2] in 2004. The NOD2 protein functions as an intracellular sensor for the bacterial cell wall product muramyl-dipeptide and is believed to play an important role in maintaining the integrity of the mucosal barrier [3]. Defective NOD2 function might lead to inefficient clearance of bacterial pathogens, resulting in subsequent hyperinflammation, which in turn promotes the development of aGVHD. The results of retrospective analyses are somewhat contradictory,

From the [1]University Hospital Carl-Gustav-Carus, Department of Medicine I Dresden, Germany; and [2]DKMS-LifeScience Lab, Dresden, Germany.

Financial disclosure: See Acknowledgments on page 1727.

Correspondence and reprint requests: Dr. Martin Wermke, Universitätsklinikum Carl-Gustav-Carus, Medizinische Klinik I, Fetscherstraße 74, 01307 Dresden, Germany (e-mail: martin.wermke@uniklinikum-dresden.de).

Received March 1, 2010; accepted June 1, 2010

1083-8791/$36.00
doi:10.1016/j.bbmt.2010.06.001

however, with some studies supporting *NOD2*-SNPs as a significant factor for aGVHD [2,4,5] but others not demonstrating such effects [6,7]. The importance of *NOD2* mutations seems to depend on the clinical context [8,9]; clearly, *NOD2*-SNPs alone are insufficient to adequately predict the risk for aGVHD in the general population of patients undergoing allo-SCT.

Another candidate gene that possibly affects the risk of immunologic complications after allo-SCT is the bactericidal permeability-increasing protein (*BPI*). BPI is a 55-kDa antimicrobial polypeptide that is preferentially expressed in neutrophils and to a lesser extent in mucosal epithelial cells [10]. It exerts direct antimicrobial effects, neutralizes lipopolysaccharides (LPS), and opsonates gram-negative bacteria [11]. Like NOD2, BPI is believed to be an essential part of the complex system that maintains mucosal integrity against a wide variety of commensal and pathogenic microbes. Recently, an SNP at position 645 of the BPI gene (A vs G) was associated with Crohn's disease [12]. *BPI*-SNPs also have been found to affect the risk of bronchiolitis obliterans syndrome (BOS) after allo-SCT [13]. The potential effect of *BPI*-SNPs on the development of aGVHD has not yet been studied.

Our understanding of autoimmune and inflammatory processes was recently broadened significantly by the identification of a novel subset of T helper cells, designated Th17 cells. These Th17 cells are characterized by the expression of interleukin (IL)-17 and IL-22 and induce IL-1β, IL-6, tumor necrosis factor α, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and several chemokines, thereby promoting local accumulation of inflammatory cells and a systemic inflammatory reaction. This seems to apply to aGVHD as well, given the report that lethal GVHD could be induced by in vitro-differentiated Th17 cells in mice [14]. IL-23 is an essential cytokine for the maintenance and proliferation of these cells, and, accordingly, lack of IL-23 led to less aGVHD in a murine transplantation model [15]. On the other hand, Das et al. [16] reported that IL-23 knockout protects against GVHD independent of Th17 cells in a Balb/c-B6 mouse model. Data in humans further corroborate the association of the IL-23 pathway with GVHD by demonstrating that individuals bearing an SNP in the IL-23 receptor (*IL-23R*) gene are less likely to experience aGVHD after allo-SCT [17].

In the present study, we investigated the role of SNPs in the genes coding for NOD2, BPI, and IL-23R as predictors for the occurrence of aGVHD after allo-SCT. We chose these loci because all have been associated with the occurrence of inflammatory bowel disease, which in many ways serves as a model for the propagation of aGVHD at the host-microbial interface. Because GVHD often correlates with graft-versus-malignancy effects as well as with TRM, we also investigated the role of these genetic variants in relapse and overall survival (OS). We retrospectively screened for SNPs in these 3 genes in a cohort of 304 patients and their respective donors.

PATIENTS AND METHODS

Patients and Donors

All patients who underwent transplantation at our center, between November 2001 and January 2006, were eligible for this retrospective analysis. Of a total of 455 patients, 108 had to be excluded because of insufficient patient and/or donor material. Furthermore, to homogenize our study cohort, 43 individuals were excluded because of incomplete clinical data, haploidentical transplantation, ex vivo T cell-depletion strategies, or nonstandard indications for transplantation (eg, metabolic diseases, solid tumors). There were no relevant demographic differences between included and excluded patients, and both cohorts consisted only of individuals of central European, Caucasian origin. Further details about the excluded patients are provided as supplementary material (Table S1).

In total, the study cohort comprised 304 patients who underwent allo-SCT from an HLA-matched related donor or from an at least 8/10 HLA-matched unrelated donor. In patients receiving a graft from a matched related donor, HLA matching was based on low-resolution (2-digit) typing for HLA-A, -B, and -C and on high-resolution typing (4-digit) for HLA-DRB1 and -DQB1. Typing in the matched unrelated setting was done in the same way until 2003, after which HLA class 1 molecules were also subjected to high-resolution typing. Details on patient and treatment characteristics are presented in Table 1. All patients received antimicrobial prophylaxis with 500 mg of ciprofloxacin per day. For myeloablative conditioning, oral metronidazole 250 mg 3 times daily was added, and patients were treated under reverse isolation from the beginning of chemotherapy. Reduced-intensity conditioning (RIC) was based mainly on fludarabine plus total body irradiation (TBI), busulfan, or melphalan and thus can be characterized as myeloablative. For standard-intensity conditioning, cyclophosphamide (120 mg/kg) in combination with either 12 Gy of hyperfractionated TBI or busulfan and etoposide plus 12 Gy TBI were the most frequently used regimens. Antithymocyte globulin (ATG Fresenius) was used for in vivo T cell depletion in most patients who received a transplant from a matched unrelated donor. GVHD prophylaxis was provided by a calcineurin inhibitor (cyclosporine A or tacrolimus) either alone or in combination with methotrexate or mycophenolate mofetil. GVHD grading was based on clinical criteria and/or histological analysis according to standard criteria [18].

Table 1. Characteristics of the 304 Patients Enrolled

Age, years, median (range)	50.8 (11-76)
Sex, n (%)	
Female	126 (41.4%)
Male	178 (58.6%)
Diagnosis, n (%)	
AML/MDS	158 (52.0%)
Lymphoma	77 (25.3%)
Myeloma	22 (7.2%)
ALL	25 (8.2%)
Myeloproliferative syndrome	17 (5.6%)
Other	5 (1.6%)
Status at transplantation, n (%)	
Compete remission, CML in chronic phase, MDS	139 (45.7%)
Advanced	165 (54.3%)
Conditioning, n (%)	
Standard	70 (23.0%)
RIC	234 (77.0%)
In vivo T cell depletion, n (%)	
Yes	96 (31.6%)
No	208 (68.4%)
Donor, n (%)	
Related	100 (32.9%)
Unrelated	204 (67.1%)
HLA match, n (%)	
Match (10/10)	231 (76.0%)
Mismatch (9/10 or 8/10)	73 (24.0%)
Donor sex, n (%)	
Female donor/male recipient	45 (14.8%)
Other	259 (85.2%)
Transplant source, n (%)	
PBSC	288 (94.7%)
BM	16 (5.3%)

All patients provided informed consent during the hospital stay. The banking of DNA for genetic studies and their correlation with clinical endpoints was approved by the local Institutional Review Board.

SNP Nomenclature

Given that published data suggest a dominant effect of *NOD2*- and *IL-23R*-SNPs on posttransplantation outcome, we focused our analysis on the dominant genetic model. Furthermore, given the low minor allele frequencies of both SNP, it would have been necessary to include far more patients than available at a single center to design an adequately powered study addressing additive or even recessive models. A dominant genetic model means that all patients and donors carrying at least one mutated allele in *NOD2* exon 8 (rs2066844), 12 (rs2066845), or 13 (rs2066847) were classified as *NOD2*-SNP. Individuals bearing only the wild-type (wt) allele at each locus were classified as *NOD2*-wt. Likewise, all individuals bearing at least one variant allele at position 1142 of the *IL-23R* gene (rs11209026) were classified as *IL-23R*-SNP, whereas those with only the wt allele were classified as IL-23R-wt. In terms of *BPI*, the presence of at least one G allele at position 645 (rs4358188) was required for classification as *BPI*-G. Individuals with two A alleles were classified as *BPI*-A under this model. Given the published frequencies of the A and G alleles, we evaluated additive and recessive models for *BPI* as well.

DNA Analysis

DNA analysis was done without knowledge of GVHD or clinical outcome status. DNA was extracted from blood or bone marrow (BM) obtained from donors and recipients before transplantation using a silica-based procedure (Qiagen DNA Blood Kit; Qiagen, Hilden, Germany) and frozen until analysis. Whole genome amplification was performed using the GenomiPhi V2 DNA amplification kit (GE Healthcare, Munich, Germany) according to the manufacturer's recommendations.

Real-time polymerase chain reaction (PCR) genotyping for *NOD2*-SNP8, -SNP12, and -SNP13, *IL-23R*-SNP, and *BPI*-SNP was done with a Lightcycler 480 (Roche, Mannheim, Germany). Primers, probes, and Taqman Universal PCR master mix were purchased from Applied Biosystems (Applera Deutschland, Darmstadt, Germany). Probes were labeled with either FAM or VIC and a nonfluorescent quencher. The final 25-μL reaction mix contained 12.5 μL of Taqman Universal PCR master mix (Applied Biosystems, Applera Deutschland), 1.25 μL of the predesigned or custom-made SNP assay, and 10 ng of DNA. PCR conditions were 10 seconds at 95°C, followed by 40 cycles of 15 seconds at 92°C and 1 minute at the annealing/extension temperature (60°C for *NOD2*-SNP12, *NOD2*-SNP13, and *BPI* and 62°C for *NOD2*-SNP8 and *IL-23R*-SNP). For quality control, specimens with a known genotype at each analyzed locus and no-template controls were included in every run of whole genome amplification and real-time PCR.

Statistical Analysis

Because of the low expected frequencies of the variant allele, the exact test as described by Wigginton et al. [19] was used to assess Hardy-Weinberg equilibrium. For time-dependent variables, patients were censored at the time of last follow-up. aGVHD was defined as any GVHD occurring before day 100. Day +100 incidences are reported. The cumulative incidence of aGVHD was analyzed by competing-risk analysis using Gray's test for comparison of groups. For that purpose, GVHD was classified as either clinically relevant (grade II-IV) or severe (grade III-IV) and compared with no or uncertain GVHD (grade 0-I) and no or nonsevere GVHD (grade 0-II), respectively. Death without GVHD was treated as a competing event. Multivariate analysis of the aGVHD rates was performed using an ordinal logit regression model to account for the varying clinical and prognostic implications of the different aGVHD grades. The proportionality assumption was checked by allowing the coefficients of the independent variables to vary for

different logits, and then testing for differences among these coefficients. Advanced disease was defined as a disease not in complete remission or not having chronic myelogenous leukemia in chronic phase or myelodysplastic syndrome (MDS). Relapse incidence was estimated using competing events statistics as described before, with death as a competing event. Cox regression was applied to search for biologic factors associated with the risk of relapse. OS was assessed using the Kaplan-Meier method, with death from any cause as the endpoint. The log-rank test was used for comparisons among the different groups. Statistical analyses were performed with R version 2.8.1 with the cmprsk package (Cran network) and SPSS version 17.0 (SPSS, Munich, Germany).

RESULTS

In our somewhat older study cohort (median age, 51 years), RIC was used in two-thirds of cases (Table 1). Most patients received peripheral blood stem cells (PBSCs) (94.7%) from a matched unrelated donor (67.1%). The median follow-up time for living patients was 38.5 months (range, 0.7-75.5 months).

Frequencies of Individual SNPs

Minor allele frequencies for *NOD2*-SNP8, - SNP12, and -SNP13 and *IL-23R* as well as *BPI* SNP645 G allele frequency were similar to those published by the NCBI SNP project (http://www.ncbi.nlm.nih.gov/projects/SNP). Details can be found in IL-23R Tables 2 and 3.

Only 4 patients were homozygous for *NOD2*-SNP8. All other patients and donors displayed either homozygous wt or heterozygous variant genotype at the *NOD2*-SNP8, -SNP12, or -SNP13 loci. Only 1 patient and 5 donors had mutations at more than 1 of the 3 *NOD2*-SNP loci and thus could be classified as compound heterozygotes. Homozygous *IL-23R* mutations were found in only 2 of 304 recipients and in only 2 of 304 donors. In contrast, 92 of 304 recipients (30.3%) and 81 of 304 donors (26.6%) demonstrated homozygosity for the G allele at position 645 of the *BPI* gene. Tables 2 and 3 list the numbers of patients with the homozygous wt, heterozygous SNP, and homozygous SNP genotypes.

When assessing whether the observed frequencies are reconcilable with the Hardy-Weinberg equilibrium, we noticed that this was not the case for *NOD2*-SNP8 in the recipients ($P = .007$; Table 2). This is because of an excess of patients bearing the homozygous SNP genotype (n = 4). In contrast, recipient genotype frequencies for *NOD2*-SNP12 ($P = 1.0$), *NOD2*-SNP13 ($P = 1.0$), *BPI* ($P = .272$), and *IL-23R* ($P = 1.0$) were consistent with the Hardy-Weinberg equilibrium. The same applied for donor genotypes frequencies for *NOD2*-SNP8 ($P = .846$), *NOD2*-SNP12 ($P = 1.0$), *NOD2*-SNP13 ($P = 1.0$), *BPI* ($P = 1.0$), and *IL-23R* ($P = .511$).

Polymorphisms and GVHD

Overall, 110 of 304 patients (36.2%) experienced aGVHD grade II-IV with a median time to onset of 19 days (range, 6-93 days). Severe aGVHD (grade III-IV) occurred in 52 of 304 patients (17.1%) at a median of 21 days (range, 6-88 days) after allo-SCT. Gastrointestinal (GI) GVHD of any grade was diagnosed in 64 of 304 patients (21.1%) at a median of 20 days (range, 6-88 days) after allo-SCT.

NOD2 polymorphisms

The cumulative incidence of clinically relevant aGVHD (grade II-IV) was not significantly different between patients who received transplants from *NOD2*-wt and *NOD2*-SNP donors (Figure 1A) at day 100 after transplantation (37.3%; 95% CI, 31.18%-43.5% vs 32.6%; 95% CI, 21.3%-43.9%; $P = .623$). Similarly, *NOD2* recipient genotype was not found to correlate with the incidence of aGVHD grade II-IV (Figure 1B). The cumulative incidence was 37.0% in patients with wt (95% CI, 30.9%-43.1%) and 33.3% in those with variant *NOD2* genotype (95% CI, 21.3%-45.4%; $P = .601$). To further dissect the influence of *NOD2*-SNPs on the occurrence of aGVHD, we divided patients into 4 subgroups according to the *NOD2* genotype of the recipient and the donor (group 1: recipient and donor wt; group 2: recipient SNP and donor wt; group 3: recipient wt and donor SNP; group 4: recipient and donor SNP). As shown in Figure 1C, the cumulative incidence function did not differ significantly among the 4 subgroups.

Donor *NOD2* genotype did not influence the occurrence of severe aGVHD (donor *NOD2*-wt:

Table 2. Genotype and Allele Frequencies of the Analyzed Loci in the Recipients

	Wt/Wt, n (%)	Wt/Variant, n (%)	Variant/Variant, n (%)	f(wt)	f(variant)
NOD2 SNP8	278 (91.4%)	22 (7.2%)	4 (1.4%)	0.95	0.05
NOD2 SNP12	290 (95.4%)	14 (4.6%)	0	0.98	0.02
NOD2 SNP13	283 (93.1%)	21 (6.9%)	0	0.97	0.03
BPI	71 (23.4%)	141 (46.4%)	92 (30.2%)	0.47	0.53
IL-23R	256 (84.2%)	46 (15.1%)	2 (0.7%)	0.92	0.08

f(wt or variant) indicates wt or variant allele frequency; BPI, bactericidal permeability-increasing.

Table 3. Genotype and Allele Frequencies of the Analyzed Loci in the Donors

	Wt/Wt, n (%)	Wt/Variant, n (%)	Variant/Variant, n (%)	f(wt)	f(variant)
NOD2 SNP8	272 (89.5%)	32 (10.5%)	0	0.95	0.05
NOD2 SNP12	289 (95.1%)	15 (4.9%)	0	0.98	0.02
NOD2 SNP13	278 (91.4%)	26 (8.6%)	0	0.96	0.04
BPI	70 (23.0%)	153 (50.3%)	81 (26.7%)	0.48	0.52
IL-23R	271 (89.1%)	31 (10.2%)	2 (0.7%)	0.94	0.06

f(wt or variant) indicates wt or variant allele frequency; BPI, bactericidal permeability-increasing.

17.4%, 95% CI, 12.5%-22.2% vs *NOD2*-SNP: 16.2%, 95% CI, 7.4%-25.1%; $P = .938$) (Figure 1D), whereas recipients bearing the *NOD2* variant genotype tended to have less aGVHD grade III-IV compared with those with the *NOD2*-wt genotype (10%; 95% CI, 2.3%-17.7% vs 18.9%; 95% CI, 13.9%-23.9%; $P = .104$) (Figure 1E). Again, an integrated analysis of recipient and donor genotype found no significant differences (Figure 1F).

Because *NOD2* variants have been reported to correlate especially closely with the risk for GI GVHD, we also assessed this endpoint in our cohort. The cumulative incidence of GI aGVHD of any grade was not associated with donor *NOD2* genotype (Figure S1A). In contrast, there was a trend toward less GI GVHD in recipients bearing a *NOD2*-SNP compared with those with the *NOD2*-wt genotype (11.7%; 95% CI, 3.5%-19.9% vs 23.4%; 95% CI, 18.0%-28.7%; $P = .052$) (Figure S1B).

Holler et al. [2] reported a significant association of *NOD2*-SNPs with GVHD when comparing patient and donor pairs with unmutated *NOD2* and those with SNPs in the donor and/or recipient. Even under these premises, we found no correlation of *NOD2* polymorphisms with the incidence of aGVHD grade II-IV (Figure S2A) and grade III-IV (Figure S2B).

BPI polymorphisms

Recipients of a transplant from a *BPI*-A donor had a higher cumulative incidence of aGVHD grade II-IV than recipients of a *BPI*-G transplant (41.8%; 95% CI, 30.1%-53.5% vs 34.6%; 95% CI, 28.5%-40.7%), but the cumulative incidence functions did not differ significantly, as assessed by Gray's test ($P = .222$) (Figure 2A).

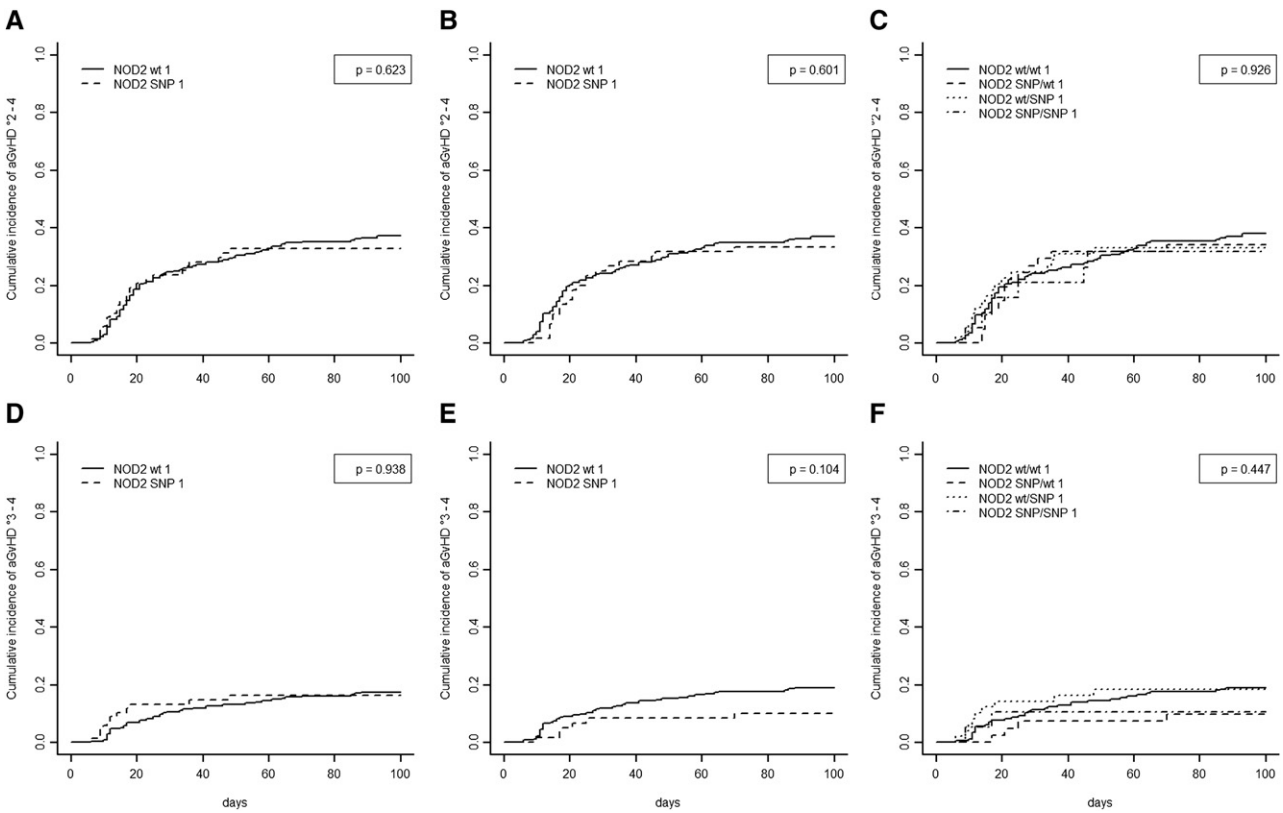

Figure 1. Cumulative incidence of aGVHD with respect to NOD2 genotype. (A and B) Incidence of aGVHD grade II-IV according to NOD2 genotype of the donor (A) and the recipient (B). (C) An integrated analysis according to donor and recipient genotype. (D and E) Cumulative incidence of severe aGVHD according to NOD2 donor genotype (D) and recipient genotype (E). (F) An integrated analysis of donor and recipient genotype and severe aGVHD. In (C) and (F), recipient genotype is given before that of the donor (eg, "wt/SNP" refers to WT in the recipient and SNP in the donor).

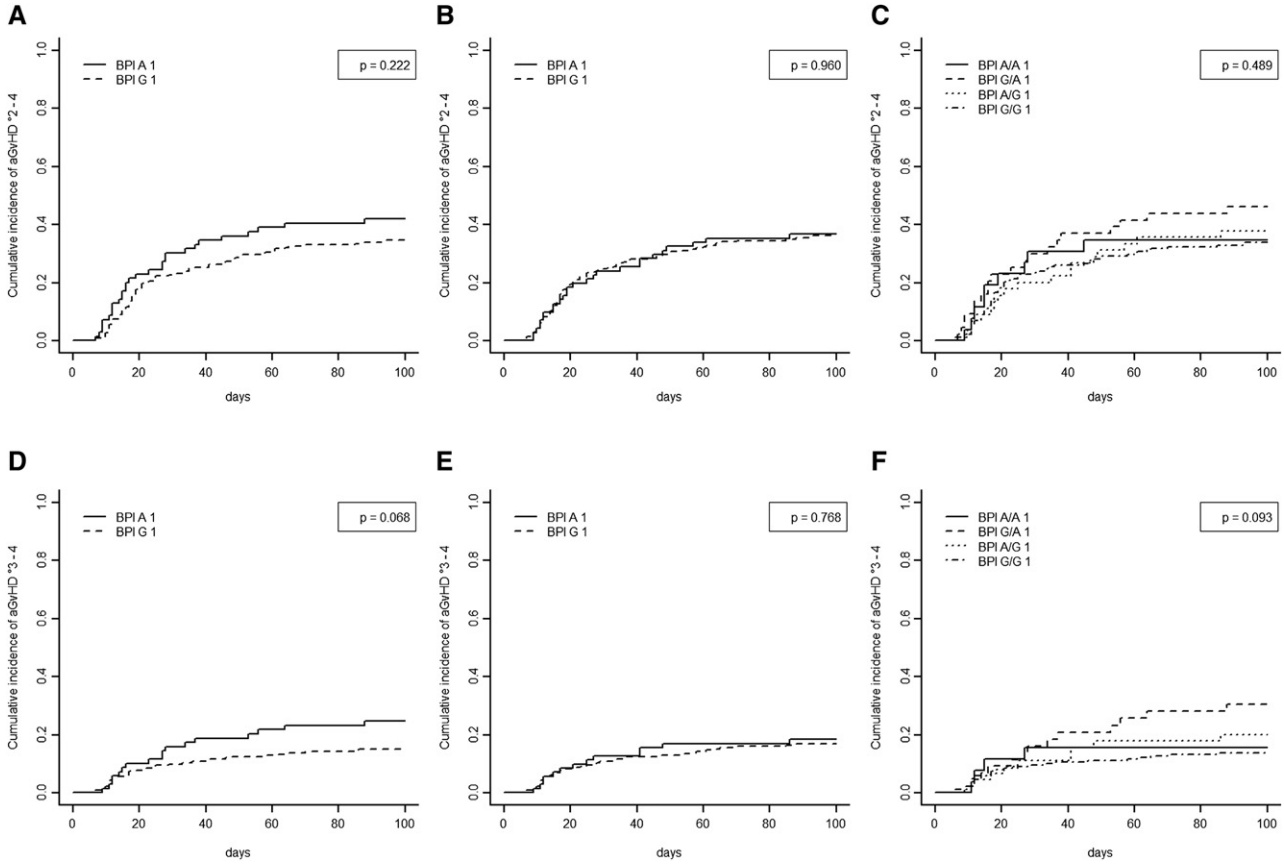

Figure 2. Correlation of BPI genotype with the incidence of aGVHD. No significant differences in the incidence of aGVHD grade II-IV could be found with respect to BPI donor genotype (A) and BPI recipient genotype (B). (C) An integrated analysis of aGVHD grade II-IV incidence according recipient and donor genotype. (D and E) Cumulative incidence of aGVHD grade III-IV according to donor genotype (D) and recipient genotype (E). (F) An integrated analysis of donor and recipient genotype and aGVHD grade III-IV. In (C) and (F), recipient genotype is given first, followed by donor genotype (eg, "A/G" means A in the recipient and G in the donor).

Furthermore, there was no relevant difference in aGVHD incidence with respect to patient *BPI* genotype (*BPI*-A, 36.6%; 95% CI, 25.3%-47.9% vs *BPI*-G: 36.1%, 95% CI, 30.0%-42.3%; $P = .960$) (Figure 2B). Subgroup analysis incorporating recipient and donor genotype revealed no marked differences (Figure 2C). However, there was a trend ($P = .068$) toward less aGVHD grade III-IV in recipients of a *BPI*-G transplant compared with recipients of a *BPI*-A transplant (15.0%; 95% CI, 10.4%-19.5% vs 24.6%; 95% CI, 14.4%-34.9%). Details of the cumulative incidence functions are shown in Figure 2D. Again, recipient *BPI* genotype was not correlated with severe aGVHD (*BPI*-A: 18.3%; 95% CI, 9.2%-27.3% vs *BPI*-G: 16.8%; 95% CI, 12.0%-21.6%; $P = .768$) (Figure 2E). An integrated analysis of recipient and donor genotypes showed that recipients with the *BPI*-G genotype receiving a *BPI*-A graft were the most likely of all groups to experience aGVHD grade III-IV (Figure 2F).

When analyzed under a recessive model, no significant associations were found between aGVHD grade II-IV and *BPI* donor (Figure S3A) or recipient genotype (Figure S3B). Similarly, analysis under these premises revealed no association between severe aGVHD (grade III-IV) and *BPI* donor (Figure S3C) or recipient genotype (Figure S3D). Assessment under an additive genetic model also failed to identify a correlation between *BPI* genotype and aGVHD (data not shown).

IL23R polymorphisms

Recipients of transplants from donors with an *IL-23R*-SNP had a cumulative aGVHD incidence of only 21.4% (95% CI, 7.1%-35.8%), compared with 38.0% (95% CI, 32.2%-43.8%) in those receiving an *IL-23R*-wt graft ($P = .089$) (Figure 3A). In contrast, recipient *IL-23R* genotype was not correlated with the occurrence of aGVHD grade II-IV, with almost equal cumulative incidence in the two groups (wt: 36.4%; 95% CI, 30.5%-42.3% vs SNP: 35.4%; 95% CI, 21.7%-49.1%; $P = .795$) (Figure 3B).

As shown in Figure 3C, patients receiving an *IL-23R*-SNP graft were less likely to develop aGVHD regardless of recipient genotype. However, this difference did not reach statistical significance for the comparison of all 4 subgroups (Figure 3C). Nevertheless, the curves suggest that if there is any effect of

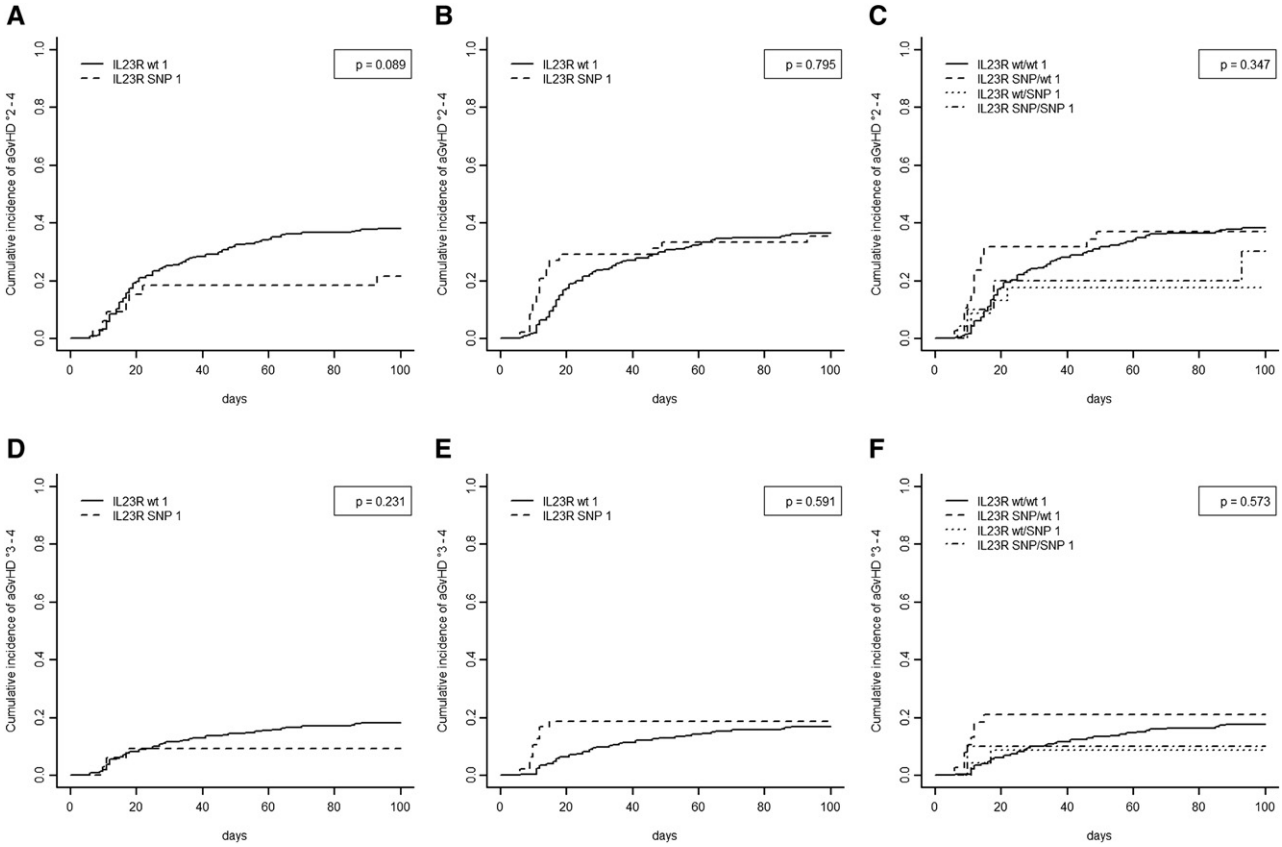

Figure 3. Cumulative incidence of aGVHD with respect to IL-23R-genotype. (A and B) IL-23R-SNP in the donor was correlated with a reduced incidence of aGVHD grade II-IV (A), whereas no differences were seen with respect to IL-23R-genotype in the recipient (B). (C) Detailed subgroup analysis revealed a lower incidence of aGVHD grade II-IV in recipients of allo-SCT from an IL-23R-SNP donor irrespective of patient genotype. (D) The incidence of aGVHD grade III-IV according to IL-23R donor genotype. (E) The incidence of aGVHD grade III-IV according to recipient IL-23R genotype. (F) An integrated analysis of IL-23R donor and recipient genotype. In (C) and (F), recipient genotype is given first, followed by donor genotype.

IL-23R-SNP, then donor genotype should be the key determinant.

The cumulative incidence of severe aGVHD (grade III-IV) in recipients of an *IL-23R*-SNP graft was only half that of recipients of an *IL-23R*-wt graft (9.1%; 95% CI, 0%-19.1% vs 18.1%; 95% CI, 13.5%-22.7%). This difference did not reach statistical significance ($P = .231$), however, presumably because of the low overall incidence of severe aGVHD (Figure 3D). In contrast, the cumulative incidence of severe aGVHD was not correlated with *IL-23R*-patient genotype (wt: 16.9%; 95% CI, 12.2%-21.5% vs SNP: 18.8%; 95% CI, 7.6%-29.9%; $P = .591$) (Figure 3E). The integrated subgroup analysis of recipient and donor genotype revealed no significant difference for the overall comparison, but again the cumulative incidence of severe GVHD was lowest in any scenario involving an *IL-23R*-SNP graft (Figure 3F).

Multivariate Analysis of Factors Influencing GVHD Incidence

Multivariate analysis of the occurrence of aGVHD was performed using an ordinal regression model. For that purpose, aGVHD was grouped into 3 strata: (1) aGVHD grade 0-I, (2) aGVHD grade II, and (3) severe aGVHD (grade III-IV). These strata were chosen to reflect the clinical implications of the different aGVHD grades. Grades 0 and I typically refer to patients with clinically irrelevant or uncertain aGVHD; most clinicians do not treat these patients systemically. Grade II usually reflects the point at which corticosteroid therapy is initiated, whereas patients with aGVHD grade III and IV have a high probability of dying from this complication and thus are often treated more aggressively. In vivo T cell depletion, donor type (related vs unrelated), type of GVHD prophylaxis (calcineurin inhibitor only vs calcineurin inhibitor plus other immunosuppressant), sex match (female donor and male recipient vs other), HLA match (10/10 match vs other), donor IL-23R genotype (wt vs SNP), recipient *NOD2* genotype (wt vs SNP), and donor *BPI*-A645G-SNP (A vs G) were entered as factors potentially influencing the incidence of aGVHD. Only in vivo T cell depletion (hazard ratio [HR], 0.35), use of a multiagent GVHD prophylaxis regimen (HR, 0.53), and donor *IL-23R*-SNP (HR, 0.40) were identified as independent factors correlated with a reduced risk of aGVHD. Furthermore, there was a trend toward less aGVHD in recipients of grafts from related donors (HR, 0.64) and in

Table 4. Multivariate Analysis of Factors Influencing the Severity of aGVHD

	OR	95% CI	*P* Value
In vivo T cell depletion, yes versus no	0.32	0.17-0.60	<.001
Female donor and male recipient versus other	1.62	0.86-3.06	.133
Related donor versus unrelated donor	0.77	0.44-1.34	.351
GVHD prophylaxis multiagent versus calcineurin inhibitor only	0.51	0.27-0.95	.033
NOD2 patient SNP versus wt	0.83	0.45-1.53	.554
IL-23R donor SNP versus wt	0.39	0.16-0.95	.039
BPI donor G allele versus A allele	0.60	0.35-1.04	.067
HLA match versus mismatch	0.57	0.32-1.21	.058

Ordinal regression modeling was used. Acute GVHD was grouped into 3 strata: (1) grade 0-I, corresponding to no or uncertain disease; (2) grade II, marking the threshold for clinically relevant aGVHD; and (3) severe GVHD, corresponding to grade III-IV.

recipients of grafts from donors with the *BPI*-G genotype (HR, 0.59). Recipient *NOD2* genotype and sex-mismatched transplant were not independent factors for the occurrence of aGVHD. Details of the analysis are presented in Table 4.

Polymorphisms and Clinical Outcome

Given the clear correlation between GVHD and graft-versus-malignancy effects, we next investigated whether any of the analyzed SNPs were correlated with relapse. At 5 years, patients with an SNP at the *NOD2* locus had a cumulative relapse incidence of 46.2% (95% CI, 32.4%-60.0%), compared with 33.2% (95% CI, 26.0%-40.3%) in those recipients with the wt *NOD2* genotype, which is a statistically significant difference ($P = .020$). In contrast, there was no difference in the 5-year cumulative incidence of relapse between *IL-23R*-wt (35.1%; 95% CI, 28.3%-41.9%) and *IL-23R*-SNP recipients (38.8%; 95% CI, 21.7%-56.0%; $P = .475$). Interestingly, relapse incidence also did not differ with respect to *IL-23R* donor genotype (SNP: 34.6%; 95% CI, 17.6%-51.5% vs wt: 35.9%; 95% CI, 29.1%-42.7%; $P = .735$). Furthermore, there was no relevant difference in incidence between the different *BPI* recipient genotype groups (A: 32.5%; 95% CI, 21.1%-43.9% vs G: 36.4%; 95% CI, 29.1%-43.8%; $P = .990$) or donor genotype groups (A: 27.3%; 95% CI, 16.4%-38.2% vs G: 37.7%; 95% CI, 30.5%-44.9%; $P = .396$). Cox regression analysis identified advanced disease status at allo-SCT as the only significant independent risk factor for relapse. *NOD2*-SNP in the recipient was an independent factor of borderline significance (Table 5). The estimated 5-year OS was 34.2% in recipients with an *NOD2*-SNP, compared with 44.3% in those with *NOD2*-wt ($P = .158$). Survival differences based on *NOD2* donor genotype were even less remarkable, with a 5-year OS of 42.7% in wt versus 41.6% in SNP ($P = .300$). The same applied for recipient *IL-23R* genotype (5-year OS: 42.6% in wt vs 40.6% in SNP; $P = .584$) and donor *IL-23R* genotype (5-year OS: 42.9% in wt vs 38.4% in SNP; $P = .732$). Recipient *BPI* genotype also had no influence on OS (A, 36.5% vs G, 44.2%; $P = .560$). Finally, *BPI* donor genotype was not associated with OS (A, 44.7% vs G, 42.5%; $P = .928$).

Table 5. Multivariate Analysis of Factors Influencing Relapse Incidence

	OR	95% CI	*P* Value
Disease status at transplantation, advanced versus early	2.13	1.37-3.29	.001
In vivo T cell depletion, yes versus no	1.23	0.81-1.87	.337
NOD2 patient SNP versus wt	1.56	0.99-2.46	.056
IL-23R donor SNP versus wt	0.88	0.46-1.71	.710
BPI donor G allele versus A allele	1.44	0.85-2.44	.178

Cox regression modeling was used. Advanced disease is defined as any disease not in complete remission or chronic phase or the presence of MDS at time of transplantation.

DISCUSSION

In our analysis of a cohort of 304 patients and their related and unrelated donors, we were unable to demonstrate a clear effect of *NOD2*-SNPs on the incidence of aGVHD. We did find a trend toward less aGVHD in patients bearing an SNP at the *NOD2* locus, but this effect could not be confirmed in multivariate analysis. Nevertheless, we found a higher relapse rate in this group, with the difference almost reaching statistical significance in multivariate analysis. In contrast, we identified a trend toward less aGVHD in recipients of a graft from an *IL-23R*(1142A>G)-SNP donor in univariate analysis, and this association reached statistical significance when assessed in a multivariate model. Despite this, these patients did not demonstrate a higher relapse rate. Interestingly, we saw a trend toward less aGVHD in patients receiving a graft from a donor with the G allele at position 645 of the *BPI* gene in both the univariate and multivariate analyses; however, this also did not result in an increased relapse rate.

Our results regarding *IL-23R* and *BPI* might be limited by the fact that we did not adjust for multiple comparisons. On the other hand, our intent was to identify potential candidates for further prospective evaluation, and thus we were reluctant to prematurely exclude any SNP because of overly conservative significance thresholds.

Regarding *NOD2*-SNP8 in the recipient, we must acknowledge that the observed genotype frequencies were not reconcilable with the Hardy-Weinberg equilibrium. This resulted mainly from an excess of 4 patients homozygous for this SNP. There are several possible explanations for this surplus. Because the experiments were performed with rigid quality control, we can largely exclude genotyping errors. Another explanation might be the use of a stratified study cohort (eg, a cohort comprising 2 or more different subpopulations); however, if that were so, then we would expect other SNPs to be in disequilibrium as well. Random

sampling effects may also account for this observation. Moreover, because 3 of these 4 patients suffered from acute myelogenous leukemia, it also is possible that loss of heterozygosity, a common phenomenon in such highly genetically instable diseases, led to a loss of the wt allele.

In general, our results are compatible with the controversial conclusions reached by other groups regarding *NOD2*-SNPs in allo-SCT. First reported to predict the occurrence of aGVHD by Holler et al. [2], these genetic variants were later shown to be correlated with relapse incidence, but not with GVHD, in a different cohort by Mayor et al. [9]. Some other authors failed to demonstrate any importance of *NOD2*-SNPs in the posttransplantation setting, however [6,7]. It has been speculated that the effect of *NOD2*-SNPs on the occurrence of GVHD is more pronounced in matched sibling transplantations, but diminished by the use of broad-spectrum antibiotic prophylaxis [2]. Because antibiotic prophylaxis in our patients was directed mainly against gram-negative bacteria, it is unlikely that the intestinal decontamination led to the lack of correlation between aGVHD and *NOD2*-SNPs. We also addressed the possibility that the importance of *NOD2* gene variants depends on the donor type or the use of T cell-depleting strategies by adjusting our multivariate model for these factors, and again found no effect. This might be because we used RIC more frequently (in nearly 80% of patients) than other groups. NOD2 might possibly be less relevant for the occurrence of aGVHD in this scenario, where acute organ damage is limited. aGVHD tends to occur later after RIC, which points to a different pathophysiological background, probably more related to $CD8^+$ T cell responses against minor histocompatibility antigens and not directly attributable to the acute inflammatory response seen after intensive conditioning [20]. Despite this, we did find an increased relapse rate in patients bearing the *NOD2* SNP. In contrast to Mayor et al. [9], we could not relate this finding to a high proportion of cases of acute lymphoblastic leukemia, given that only 8.2% of our patients had acute lymphoblastic leukemia.

The functional consequences of *NOD2*-SNPs are as controversial as the clinical findings. It has been postulated that a defective *NOD2* gene results in increased peritransplantation mucosal damage, which might be the reason for the association with GVHD [2,8]. Interestingly, recent mouse data point to a central role for aberrantly activated host dendritic cells in this regard [1]. Other animal work has shown that *NOD2* is also essential for T helper cell function [21], which would provide an explanation for increased relapse rates in recipients of grafts from donors carrying *NOD2*-SNPs. However, we and others [9] have found an increased relapse rate in recipients with an *NOD2*-SNP. A key problem of the aforementioned mouse models is that they use homozygous knockout instead of the heterozygous SNPs occurring in humans. This is underscored by data suggesting that only double mutations (ie, the presence of 2 SNPs in one patient) lead to a marked decrease in IL-8 production in response to muramyl-dipeptide [22]. Thus, the consequences of a single defective allele might be less severe and result in phenotypic consequences only under special circumstances. The nature of these contributing factors might be elucidated in larger clinical trials or whole genome association studies.

Our findings regarding the relevance of *IL-23R*-SNP(1142A>G) corroborate data reported by Elmaagacli et al. [17], who also found a lower frequency of aGVHD in recipients of transplants from donors with an IL-23R-SNP allele. Gruhn et al. [23] recently reported a similar effect of this genetic variation in a cohort of pediatric and adolescent allo-SCT recipients. Interestingly, this study also found no effect of *NOD2* polymorphism on posttransplantation outcome. IL-23 exerts its proinflammatory effects through both the innate and adaptive immune systems [24]. In the latter, IL-23 is essential for the generation of a novel T helper cell subset, the Th17 cells, which have been shown to induce lethal GVHD in some mouse models [14]. Other researchers have reported proinflammatory properties of IL-23 independent of Th17 cells [16]. Interestingly, they found that IL-23 knockouts were selectively protected from intestinal GVHD. Therefore, they speculated that pharmacologic targeting of IL-23 might help to protect the intestine from inflammatory damage without hampering the desired graft-versus-leukemia effect. In this context, it is interesting that we did not find an increased relapse rate in our recipients of *IL-23R*-SNP transplants despite significantly less GVHD in this group. This might be a hint that defective IL-23 signaling has similar effects in mice and humans.

Comparable to *NOD2*-SNPs and the *IL-23R* (11421>G)-SNP, an association with inflammatory bowel disease has also been found for *BPI*-SNPs [12,25]. Moreover, *BPI* has been identified as a candidate locus for the prediction of BOS after allo-SCT [13], which points to a role of this protein in posttransplantation immune regulation. Given BPI's importance in maintaining an intact mucosal barrier and defense against commensal bacteria, altered BPI function in the recipient can be assumed to be especially relevant. On the other hand, BPI has potent anti-inflammatory properties, which relate to its LPS-neutralizing function [26]. In this regard, BPI is an antagonist to LPS-binding protein, which increases the inflammatory response to LPS. Neutrophils are believed to be the main source of BPI in humans [27]; thus, genetic variants influencing *BPI* expression or function in the donor could affect posttransplantation immune regulation in the recipient. The functional

relevance of the studied *BPI*(A645G)-SNP has not yet been formally proven, however. Furthermore, our results regarding this SNP did not reach statistical significance, and the integrated subgroup analysis combining recipient and donor genotype gave somewhat conflicting results. Thus, it might turn out that this genetic variant is merely a surrogate marker for another, as-yet-unknown mutation, or that the association found in our cohort is simply because of the high frequency of *BPI*-G variants. Clearly, further work is needed to address these problems, and it will be interesting to see whether other groups reach similar conclusions.

In summary, we think that it is still too early to incorporate *NOD2*-SNPs into daily practice of donor selection or patient-specific modification of the conditioning regimen. Cleary, results from ongoing prospective trials are needed before final conclusions regarding this genetic variation can be reached. Regarding the *IL-23R*(1142A>G)-SNP and *BPI*(A645G)-SNP, we consider further prospective investigation warranted. The *IL-23R*(1142A>G)SNP might be an especially promising target, possibly allowing improvement of algorithms for donor selection.

ACKNOWLEDGMENTS

Financial disclosure: The authors have nothing to disclose.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2010.06.001.

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;115,10:1865-1872.
2. Holler E, Rogler G, Herfarth H, et al. Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GVHD following allogeneic stem cell transplantation. *Blood*. 2004;104:889-894.
3. Inohara N, Ogura Y, Fontalba A, et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2: implications for Crohn's disease. *J Biol Chem*. 2003;278:5509-5512.
4. Elmaagacli AH, Koldehoff M, Hindahl H, et al. Mutations in innate immune system NOD2/CARD 15 and TLR-4 (Thr399Ile) genes influence the risk for severe acute graft-versus-host disease in patients who underwent an allogeneic transplantation. *Transplantation*. 2006;81:247-254.
5. van der Velden WJ, Blijlevens NM, Maas FM, et al. NOD2 polymorphisms predict severe acute graft-versus-host and treatment-related mortality in T-cell-depleted haematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2009;44:243-248.
6. Granell M, Urbano-Ispizua A, Arostegui JI, et al. Effect of NOD2/CARD15 variants in T-cell-depleted allogeneic stem cell transplantation. *Haematologica*. 2006;91:1372-1376.
7. Sairafi D, Uzunel M, Remberger M, et al. No impact of NOD2/CARD15 on outcome after SCT. *Bone Marrow Transplant*. 2008;41:961-964.
8. Holler E, Rogler G, Brenmoehl J, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. *Blood*. 2006;107:4189-4193.
9. Mayor NP, Shaw BE, Hughes DA, et al. Single nucleotide polymorphisms in the NOD2/CARD15 gene are associated with an increased risk of relapse and death for patients with acute leukemia after hematopoietic stem-cell transplantation with unrelated donors. *J Clin Oncol*. 2007;25:4262-4269.
10. Canny G, Levy O, Furuta GT, et al. Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia. *Proc Natl Acad Sci USA*. 2002;99:3902-3907.
11. Weiss J, Elsbach P, Olsson I, et al. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. *J Biol Chem*. 1978;253:2664-2672.
12. Klein W, Tromm A, Folwaczny C, et al. A polymorphism of the bactericidal/permeability increasing protein (BPI) gene is associated with Crohn's disease. *J Clin Gastroenterol*. 2005;39:282-283.
13. Chien JW, Zhao LP, Hansen JA, et al. Genetic variation in bactericidal/permeability-increasing protein influences the risk of developing rapid airflow decline after hematopoietic cell transplantation. *Blood*. 2006;107:2200-2207.
14. Carlson MJ, West ML, Coghill JM, et al. In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. *Blood*. 2009;113:1365-1374.
15. Thompson JS, Chu Y, Glass JF, et al. Absence of IL-23p19 in donor allogeneic cells reduces mortality from acute GVHD. *Bone Marrow Transplant*. 2010;45:712-722.
16. 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.
17. Elmaagacli AH, Koldehoff M, Landt O, et al. Relation of an interleukin-23 receptor gene polymorphism to graft-versus-host disease after hematopoietic-cell transplantation. *Bone Marrow Transplant*. 2008;41:821-826.
18. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15:825-828.
19. Wigginton JE, Cutler DJ, Abecasis GR. A note on exact tests of Hardy-Weinberg equilibrium. *Am J Hum Genet*. 2005;76:887-893.
20. Deeg HJ, Antin JH. The clinical spectrum of acute graft-versus-host disease. *Semin Hematol*. 2006;43:24-31.
21. Shaw MH, Reimer T, Sanchez-Valdepenas C, et al. T cell-intrinsic role of Nod2 in promoting type 1 immunity to. *Toxoplasma gondii. Nat Immunol*. 2009;10:1267-1274.
22. van Heel DA, Hunt KA, King K, et al. Detection of muramyl dipeptide-sensing pathway defects in patients with Crohn's disease. *Inflamm Bowel Dis*. 2006;12:598-605.
23. Gruhn B, Intek J, Pfaffendorf N, et al. Polymorphism of interleukin-23 receptor gene but not of NOD2/CARD15 is associated with graft-versus-host disease after hematopoietic stem cell transplantation in children. *Biol Blood Marrow Transplant*. 2009;15:1571-1577.
24. Hue S, Ahern P, Buonocore S, et al. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med*. 2006;203:2473-2483.
25. Petermann I, Huebner C, Browning BL, et al. Interactions among genes influencing bacterial recognition increase IBD risk in a population-based New Zealand cohort. *Hum Immunol*. 2009;70:440-446.
26. Wittmann I, Schonefeld M, Aichele D, et al. Murine bactericidal/permeability-increasing protein inhibits the endotoxic activity of lipopolysaccharide and gram-negative bacteria. *J Immunol*. 2008;180:7546-7552.
27. Levy O. A neutrophil-derived anti-infective molecule: bactericidal/permeability-increasing protein. *Antimicrob Agents Chemother*. 2000;44:2925-2931.