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

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To assess the role of *NOD2/CARD15* variants on the long-term outcome of allogeneic stem cell transplantation in a genetically homogeneous group, we extended our previous study (cohort I, n = 78) and typed DNA for *NOD2/CARD15* single nucleotide polymorphisms (SNPs) from an additional 225 recipients and their HLA-identical sibling donors (cohort II) treated at four other European centers. Results of genotyping were compared with clinical outcome. The strong association of *NOD2/CARD15* variants with transplantation-related mortality (TRM) was confirmed in univariate and multivariate analysis; TRM increased from 20% in cohort I/22% in cohort II in recipient/donor pairs without any *NOD2/CARD15* variants to 47% in cohort I/32% in cohort II in the presence of one variant in either donor or recipient and further to 57% in cohort I/74% in cohort II in the presence of 2 or more variants ($P < .002$ in both cohorts). *NOD2/CARD15* SNPs were not associated with relapse rate but had a strong impact on overall survival. In an analysis of center effects, the type of gastrointestinal decontamination was the only factor interfering with the prognostic significance of *NOD2/CARD15* SNPs. Our data further support an interaction between gastrointestinal defense mechanisms, activation of the innate immune system, and specific transplant-related complications. (Blood. 2006;107:4189-4193)

Introduction

Pathophysiologic concepts of graft-versus-host disease (GvHD) have been based on several hypotheses. Although there is no doubt that activation of donor T lymphocytes is the central event in the alloreactivity, the concomitant interaction of the innate immune system also plays an important role and has been conceptually addressed by several investigators. The findings of van Bekkum et al[1] on prevention of GvHD by breeding mice under germfree conditions initiated a longstanding discussion on the role of gnotobiotic prophylaxis and the mechanisms of interaction between microbial and specific T-cell activation. With description of cytokines as major effectors[2,3] of at least acute GvHD, the endotoxin hypothesis postulated that the translocation of bacterial toxins across the gastrointestinal mucosa gave rise to costimulation of cytokine release and an alloimmune response. However, the findings of Beelen et al,[4] demonstrating a strong effect of elimination of anaerobic bacteria on GvHD in patients, and the recent observations that mice lacking Peyer patches[5] or receiving prophylaxis with lactobacilli[6] have a strongly reduced incidence of GvHD, suggested again a more direct interaction of bacterial ligands and activation of specific immune responses.

Recently, our group reported a significant association between the occurrence of single nucleotide polymorphisms (SNPs) within the *NOD2/CARD15* gene and GvHD as well as transplantation-related mortality (TRM).[7] The *NOD2/CARD15* gene had been identified as the first "susceptibility" or "disease" gene in Crohn disease, a form of inflammatory bowel disease.[8,9] It codes for an intracytoplasmic sensor of the bacterial cell-wall compound muramyl-dipeptide (MDP). Ligation of MDP to *NOD2/CARD15* induces a signal transduction cascade finally leading to activation of the transcription factor NF-κB and subsequent inflammatory response in epithelial cells of the ileum[10] as well as in monocyte/macrophage-derived cells. Because the first series analyzed consisted of a heterogeneous group of allogeneic HLA-identical sibling and unrelated donor hematopoietic stem cell transplantations (HSCTs), it did not allow a more detailed analysis within these genetically different transplantation groups. Therefore, we now have performed an analysis focusing only on HLA-identical sibling transplantations including patients from the first cohort and a larger, second and independent cohort of patients, and addressed the impact of *NOD2/CARD15* SNPs on long-term transplantation

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outcome. The strong impact of *NOD2/CARD15* variants on acute GvHD and early TRM was confirmed and translated as a strong impact on long-term overall survival (OS). Furthermore, our data suggest an interference of *NOD2/CARD15* genotype with prophylactic use of gastrointestinal decontamination.

Patients, materials, and methods

Patients and donors

A total of 303 consecutive patients from five different centers were included in this analysis, the cohort of 78 patients from Regensburg (center 1; cohort I) had been partially included in our first single-center report on the role of *NOD2/CARD15* in allogeneic HSCTs but so far had not been analyzed for long-term outcome. To these 78 patients, 225 patients (cohort II) were added from the centers of Vienna (center 2; n = 38), Newcastle (center 3; n = 90), Rostock (center 4; n = 37), and Paris (center 5; n = 60). All patients received grafts from HLA-identical sibling donors. Patients had been treated according to center-specific protocols of conditioning regimens, immunosuppressive prophylaxis, and gastrointestinal decontamination. Major patient characteristics and outcome variables were reported and analyzed based on uniform case record forms (CRFs). In all centers, patients and their HLA-identical sibling donors gave informed consent approved by local ethics review boards to analyze genetic risk factors associated with GvHD and outcome following allogeneic SCT. Approval was obtained from the University of Regensburg Medical Center's and from the other participating centers' Institutional Review Board for these studies. Detailed characteristics of patients and transplantation-specific strategies are given in Table 1. Strategies of gastrointestinal decontamination were grouped according to efficacy against Gram-positive bacteria. Type 1 decontamination included no decontamination at all or decontamination addressing mainly Gram-negative and anaerobic bacteria (ciprofloxacin and late metronidazole starting from day +1); type 2 decontamination also covered Gram-positive bacteria either via the use of amoxicillin and ciprofloxacin (center 5) or via oral vancomycin and nonabsorbable polymyxins (center 2). The median follow-up time was 30 months (range, 1-220 months) in surviving or censored patients and 6 months (range, 0-36 months) in patients dying from complications.

Typing for *NOD2/CARD15* SNPs

Prior to admission, DNA from recipients and their respective donors had been isolated from EDTA blood and stored until central analysis at the University of Regensburg. In some centers, EBV cell lines from donors and recipients had been established and DNA was isolated from cryopreserved EBV cell lines. *Taq*Man polymerase chain reaction (PCR) for SNPs 8, 12, and 13 of the *NOD2/CARD15* gene was performed as previously described.[11]

Analysis of data

An SPSS 12.0 database was established based on the CRFs, which included the typing results for *NOD2/CARD15* and was used for analysis of frequencies, survival data, and risk factors. Cox regression analysis for TRM and OS included *NOD2/CARD15* status, cohort and center, age, status of disease at the time of HSCT, sex combination (female donor in male recipients versus others), intensity of conditioning (standard versus reduced intensity), stem cell source, and type of gastrointestinal decontamination. The *NOD2/CARD15* status of the patients was grouped according to the number of *NOD2/CARD15* variants observed in an individual donor-recipient pair: wild-type was absence of any variant in recipient and donor; one variant was one heterozygous variant in either recipient or donor; 2 or more meant compound heterozygous or homozygous variants in recipients (n = 1) or donors (n = 1) or in both (n = 26).

Significance testing was performed by using the likelihood ratio test ($P < .05$). For calculation of TRM and incidence of GvHD and relapse, the cumulative incidence method with either relapse or non–relapse-related mortality treated as competing risks was used with the help of NCSS 2004 software (NCSS, Kaysville, UT). All group comparisons were done using the Fisher exact test. $P < .05$ was considered as statistically significant.

Results

Distribution of *NOD2/CARD15* variants and number of *NOD* variants in the transplantation cohorts

As shown in Table 2, the distribution of *NOD2/CARD15* variants within recipients and donors as well as the distribution of the number of *NOD2/CARD15* variants were similar in both cohorts of patients.

Table 1. Characteristics of patients according to transplantation centers and cohorts

	Cohort I Center 1	Cohort II Center 2	Cohort II Center 3	Cohort II Center 4	Cohort II Center 5	*P*
No. of patients	78	38	90	37	60	—
Female/male	28/50	22/16	36/54	11/26	24/36	NS
Female D and male R	29%	15%	34%	19%	30%	NS
Median age at HSCT, y (range)	47 (17-64)	43 (21-60)	36 (10-57)	43 (16-66)	34 (3-66)	.001
Underlying disease						.006
Acute leukemia	49%	49%	49%	46%	42%	
Chronic leukemia	14%	15%	27%	30%	40%	
BMF	1%	9%	21%	0%	10%	
Lymph neopl	36%	27%	3%	24%	8%	
Early or intermediate disease status at transplantation	62%	75%	83%	78%	88%	.001
Type of GI decontamination						.001
None or G− (1)	100%	—	100%	100%	—	
G+ and G− (2)	—	100%	—	—	100%	
Standard therapy	49%	56%	58%	68%	81%	.002*
RIC	51%	44%	42%	32%	19%	—
Marrow	19%	12%	65%	46%	64%	.001†
PBSCs	81%	88%	35%	54%	36%	—

No major differences with regard to prophylactic immunosuppression were observed, and T-cell depletion was used only in a minority of patients and centers.

NS indicates not significant; D, donor; R, recipient; BMF, bone marrow failure syndromes (severe aplastic anemia, Fanconi anemia, paroxysmal nocturnal hemoglobinuria); lymph neopl, lymphatic malignancies including non-Hodgkin lymphomas, Hodgkin disease, and myeloma; GI, gastrointestinal; G−, Gram-negative; G+, Gram-positive; and —, not applicable.

*Comparison of standard therapy and RIC.

†Comparison of marrow and PBSCs.

Table 2. Distribution of major *NOD2/CARD15* variants according to cohorts analyzed

NOD2/CARD15	Cohort I, n = 78	Cohort II, n = 225
Wild type	71%	74%
1 variant		
R variant only	11%	8%
D variant only	8%	9%
2 or more variants		
R and D variant	10%	8%
Homozygous	—	1%

P by Fisher exact test was not significant.
— indicates not observed.

Effect of *NOD2/CARD15* variants on GvHD III/IV, 1-year TRM, and overall TRM in both cohorts

When we checked the impact of *NOD2/CARD15* variants on major outcome variables, similar associations were observed in both cohorts. Although the relative increases in incidences seemed to be more pronounced in cohort I as compared to cohort II, results were uniformly significant for all major parameters (Table 3) including GVHD III/IV, gastrointestinal GvHD, 1-year TRM, and overall TRM. Causes of death were comparable in both cohorts. Death could be directly attributed to GvHD in 24%, to major bacterial or viral infection in 19%, to fungal infections in 10%, to primary or secondary respiratory failure in 35%, and to organ toxicities in 12%. Within patients with *NOD2/CARD15* mutations, there was a trend to a higher proportion of patients dying from respiratory failure (data not shown).

Multivariate Cox regression analysis including major known risk factors such as older age (> 40 years), diagnosis, status of disease at the time of HSCT, sex combination, type of gastrointestinal decontamination, intensity of conditioning, and stem-cell source confirmed the impact of *NOD2/CARD15* status, especially for the high-risk group bearing 2 or more variants, as the only significant and independent risk factors for TRM in both cohorts of patients (Table 4).

Table 3. Number of *NOD2/CARD15* variants and outcome in both cohorts of patients

	Wild-type, %	One variant, %	Two or more variants, %	*P*
GvHD III/IV				
Cohort I	12	46	57	.001
Cohort II	14	19	35	.04
GI GvHD stage II–IV				
Cohort I	13	40	43	.02
Cohort II	8	32	36	.004
TRM at 1 y				
Cohort I	15	47	57	.001
Cohort II	13	22	36	.04
Overall TRM				
Cohort I	20	47	57	.002
Cohort II	22	32	74	.002
OS				
Cohort I	59	15	0	.001
Cohort II	53	47	21	.02

Frequencies are given for severe GvHD and severe gastrointestinal GvHD, cumulative incidences (with relapse-related mortality as competing risk) for 1-year and overall TRM and Kaplan Meier estimates for overall survival OS. GI indicates gastrointestinal.

Table 4. Multivariate risk factor analysis for overall TRM

No. of *NOD2/CARD15* variants	HR	95% CI	*P*
Cohort I			
Wild-type	1.0	NA	.04
1 variant	2.7	0.9-7.8	.07
2 or more variants	5.9	1.3-27.3	.04
Cohort II			
Wild-type	1.0	NA	.002
1 variant	1.7	0.8-3.6	.17
2 or more variants	3.9	1.8-8.6	.001

The number of *NOD2/CARD15* variants is the only significant risk factor for TRM in both cohorts of patients.
NA indicates not applicable.

Long-term effects: association of *NOD2/CARD15* variants with TRM demonstrates a strong impact on OS

In contrast to TRM, *NOD2/CARD15* status had no major impact on relapse incidence and relapse-related mortality. For all patients, cumulative incidence of relapse-related mortality was 14% (95% CI, 10%-20%) in recipient and donor without *NOD* variants, 19% (95% CI, 11%-36%) in recipient and donor with one heterozygous variant, and 11% (95% CI, 4%-32%) in transplants with 2 or more variants. In line with this, the incidence of overall chronic GvHD at 1 year after SCT showed only a trend for a higher rate of chronic GvHD in *NOD2/CARD15* variants but did not reach statistical significance (data not shown).

Thus, association of *NOD2/CARD15* status with TRM demonstrated a strong and highly significant impact on OS for the whole group of patients (Figure 1) and also for individual cohorts (wild-type cohort I 60%, cohort II 53%; 1 variant: cohort I 15%, cohort II 47%; 2 or more variants: cohort I 0%, cohort II 21%; log rank for cohort I, $P < .001$; for cohort II, $P < .02$). In multivariate analysis, *NOD2/CARD15* status remained the only significant factor associated with survival in both cohorts whereas advanced stage was of significance (HR 2.67; 95% CI, 1.18-5.96) only in cohort I (Table 5).

When OS was analyzed in relation to the occurrence of isolated or combined recipient or donor variants, a similar pattern as seen for TRM in the first report was observed. Whereas 221 patients with wild-type *NOD2/CARD15* of recipients and donors had an OS rate of 56%, a strong impact of either recipient (n = 27, OS 23%, $P = .005$) or simultaneous recipient and donor variants (n = 26, OS 17%, $P = .001$) was seen. If only the donors possessed *NOD2/CARD15* SNPs, then OS was intermediate (40%, no significant difference from the wild-type group).

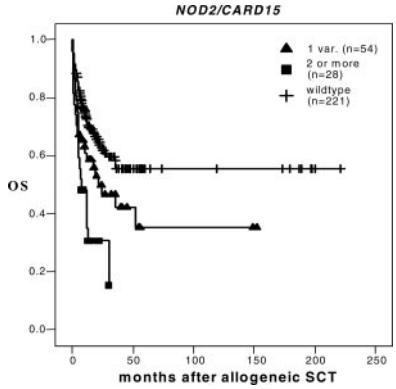

Figure 1. Overall survival according to the number of *NOD2/CARD15* variants. $P = .02$ for one variant versus wild-type, $P < .06$ for 2 variants versus one variant, $P \leq .001$ for 2 variants versus wild-type (Kaplan Meier, log rank).

Table 5. Multivariate risk factor analysis for overall long-term survival

	HR	95% CI	*P*
Advanced stage at HSCT			
Cohort I	2.67	1.18-5.96	.018
Cohort II	0.89	0.49-1.59	NS
No. of *NOD2/CARD15* variants			
Cohort I			
Wild type	1.0	NA	Overall .001
1 variant	2.8	1.30-5.87	.008
2 or more variants	3.9	1.47-10.46	.007
Cohort II			
Wild type	1.0	NA	Overall .012
1 variant	1.2	0.63-2.12	NS
2 or more variants	2.7	1.39-5.1	.003

The number of *NOD2/CARD15* variants is the main significant risk factor for OS in multivariate analysis of both cohorts.

NA indicates not applicable; NS, not significant.

Center effects may be explained by the type of gastrointestinal decontamination

Because associations of *NOD2/CARD15* seemed to be less stringent in cohort II when compared to our first series, we checked for the prognostic role of *NOD2/CARD15* in individual centers. Although influenced by different numbers of patients, the overall role of *NOD2/CARD15* seemed to be consistently strong in 3 centers but less clear in 2 others. Significant associations for both TRM and OS were observed in centers 1, 3, and 4, whereas minor trends occurred in centers 2 and 5 (Table 6).

Because patient and transplant characteristics showed major differences among centers, we next checked whether these factors interfered with the prognostic significance of *NOD2/CARD15*. After stratification for risk factors differing between participating centers such as age of the patient, status of disease at the time of HSCT, underlying disease, standard versus reduced-intensity conditioning, and marrow versus peripheral-blood stem cells (PBSCs) as stem-cell source, *NOD2/CARD15* remained a significant risk factor for GvHD and overall TRM in the subgroups. However, after stratification for the type of intestinal decontamination, the clear effect of *NOD2/CARD15* variants on GvHD and overall TRM was only seen in patients receiving decontamination with ciprofloxacin or no decontamination at all (as used in centers 1, 3 and 4) but was strongly reduced in patients receiving decontamination including Gram-positive bacteria (as used in centers 2 and 5; Figure 2). As a control, association of TRM and survival with the described risk factors was tested in the high-risk group bearing 2 or more NOD2/CARD15 variants only. Again, only the type of decontamination reached significance in multivariate analysis (HR for decontamination including Gram-positive bacteria 0.08 (0.01-0.79 95% CI, $P < .03$).

Table 6. Center effects for the association of the number of *NOD2/CARD15* variants and overall TRM

TRM in individual centers	Wild-type, %	One variant, %	Two or more variants, %	*P* by log rank
Center 1	20	47	57	.002
Center 2	11	25	0	NS
Center 3	21	38	100	.007
Center 4	23	33	50	.04
Center 5	33	29	44	NS

NS indicates not significant.

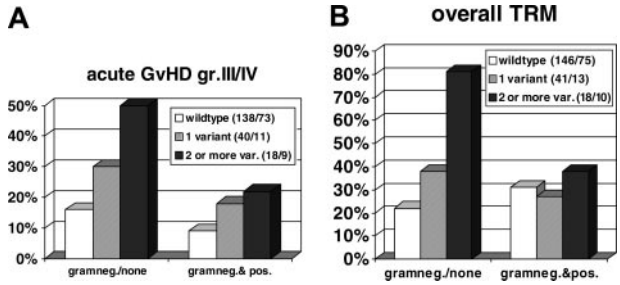

Figure 2. Major impact of the type of gastrointestinal decontamination on prognostic significance of *NOD2/CARD15* variants. Differences were highly significant ($P < .001$) for patients receiving Gram-negative decontamination or no decontamination at all, but did not reach significance in patients with combined Gram-negative and Gram-positive decontamination. Numbers in parentheses indicate the number of patients analyzed for each type of gastrointestinal decontamination. (A) GvHD grade III/IV and type of decontamination (n = 289, 14 patients were excluded from analysis due to early death). (B) Overall TRM and type of decontamination (n = 303).

Discussion

After our first report on an association of *NOD2/CARD15* variants with GvHD and TRM following allogeneic SCT, we now were able to extend our observations to a second independent cohort and to analyze for the first time a genetically homogeneous group of HLA-identical sibling transplant recipients and their respective donors from 5 different European centers. Our data not only confirm the previously reported associations in a large second cohort but also add substantial new information on the role of *NOD2/CARD15* SNPs with regard to long-term outcome, graft-versus leukemia effects, and the potential role of gene dosage and interfering factors.

Within the large and genetically homogeneous group of HLA-identical sibling transplant recipients, we analyzed the long-term effect of *NOD2/CARD15* mutations on transplantation outcome. Many strategies or factors associated with GvHD are inversely and directly linked to the major therapeutic principle of allogeneic SCT and the graft-versus-leukemia (GvL) effect, and patients with severe GvHD have a low risk of relapse and vice versa.[12,13] In contrast, analysis of survivors with *NOD2/CARD15* variants indicated an almost identical cumulative incidence of relapse in all cohorts. As a result, the *NOD2/CARD15* status had a strong and independent influence on OS. This influence was also stronger than the role of the otherwise most established clinical risk factor, the status of disease at time of HSCT. Thus, our data give rise to the hypothesis that the part of the pathophysiology of GvHD, which links the interactions of microbial load with innate immunity and the activation of the specific immune response, is not directly involved in GvL effects. This hypothesis would be in line with experimental results suggesting that modulating the inflammatory pathway induced by LPS prevents GvHD but spares GvL effects.[14] Furthermore, absence of cross-reaction between bacterial antigens in the gut and tumor antigens may also be derived from careful analysis of risks for various non–gastrointestinal cancers in patients suffering from Crohn disease because these studies did not reveal any protective effect of intestinal inflammation.[15]

So far, we do not know exactly how an impaired function of *NOD2/CARD15* may mediate GvHD and TRM. The paradigm of a (at least partial) *NOD2/CARD15* defect and an increased susceptibility to inflammation has been extensively discussed in the setting of Crohn disease,[16] because both gain of function with increased IL-1β–driven inflammation[17] and loss of function with deficient

NF-κB response, and NF-κB–dependent inflammation[18] and bacterial killing[19] have been reported in patients and mice with *NOD2/CARD15* variants. However, even given that patients with *NOD2/CARD15* variants are susceptible to uncontrolled inflammation, the cellular players are still not clear; *NOD2/CARD15*-driven inflammation might directly contribute to increased alloreactivity by delivering altered danger signals to antigen-presenting cells thus affecting T-cell activation. Alternatively, *NOD2/CARD15* variants may have a more indirect effect via a dysregulated macrophage/monocyte or epithelial response to microbial challenges.

The hypothesis of a role of specific bacterial pathogens in gastrointestinal inflammation and subsequent GvHD was raised by the reports from Beelen et al[4] on the role of elimination of anaerobic bacteria. The observed association of the type of gastrointestinal decontamination and the prognostic significance of *NOD2/CARD15* status in our present study provides some additional evidence for the role of specific pathogens, because they suggest that a Gram-positive bacterial population might be relevant for activation of *NOD2/CARD15*-mediated or -regulated inflammation. Because MDP, the specific ligand of *NOD2/CARD15*, is a bacterial cell-wall compound of both Gram-negative and Gram-positive bacteria,[20] the hypothesis seems reasonable. Of course, our conclusion is based on a relatively small number of patients and confirmation of the role of decontamination in relation to *NOD2/CARD15* variants in a larger prospective series of patients is urgently needed.

In our previous paper, we reported the strongest association of *NOD2/CARD15* variants with outcome in patients with combined donor/recipient variants. Combined donor/recipient variants may have the strongest impact on GvHD and TRM because of the additive effects of damage in affected cells, for example, recipient epithelium and donor monocytes but also represent an additive effect of mutation dose in macrophage-derived cells of the donor and the recipient. Because mutation dose of *NOD2/CARD15* has been shown to have a strong impact not only on the relative risk of Crohn disease but also on functional defects like MDP-induced IL-8 secretion,[21-23] we preferred to perform the present analysis based on the number of variants present in an individual donor/recipient pair and were able to show strong cumulative effects. However, the evaluation of the exact contribution of donor and recipient cells and the respective cellular components will require sophisticated mouse experiments addressing isolated NOD2/CARD15 deficiencies in donors and recipients and T-cell versus macrophage populations.

Clinically, it might be desirable but difficult to overcome the impact of *NOD2/CARD15* variants by altered prophylactic strategies. Selecting an alternative donor might be helpful especially if a *NOD2/CARD15* variant is present in the recipient. Reducing the epithelial damage by the use of nonmyeloablative strategies, which alter the pattern of GvHD,[24] might be another approach, although so far our analysis including standard and reduced-intensity conditioning (RIC) strategies did not show any effect of intensity of conditioning. Adapting the strategy of gastrointestinal decontamination might be a further option, but clearly prospective trials are needed to confirm the association of the type of decontamination and the prognostic role of *NOD2/CARD15* status. However, there is no doubt that these findings move forward our understanding of the clinical pathophysiology of transplantation because they demonstrate a link between alterations of innate immunity and specific posttransplantation complications. An association of the gastrointestinal defense against pathogens and GvHD has been clinically postulated for many years and now seems to find confirmation with defined genetic data. Finally, our observations raise the hope that assessment of SNPs within inflammatory or immunoregulatory pathways might indeed lead to individualized and risk-adapted clinical strategies.

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