

# Both donor and recipient *NOD2/CARD15* mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation

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Single nucleotide polymorphisms (SNPs) of the *NOD2/CARD15* gene resulting in a diminished nuclear factor- $\kappa$ B (NF- $\kappa$ B) response to bacterial cell wall products have been associated with an increased incidence of Crohn disease. To assess a possible contribution of *NOD2/CARD15* mutations to graft-versus-host disease (GvHD) and complications following allogeneic stem cell transplantation, we retrospectively typed DNA from donor/recipient pairs in 169 consecutive patients receiving transplants from related or unrelated donors. Mutated alleles were ob-

served in 21% of patients and in 14% of donors. Cumulative incidence of 1-year, transplant-related mortality rose from 20% in donor/recipient pairs without mutated SNPs to 49% in pairs with recipient mutations ( $P = .03$ ) and 59% in pairs with donor mutations ( $P < .005$ ), and was highest in 12 pairs with mutated alleles in both donor and recipients (83%;  $P < .001$ ). Similar associations were observed for severe overall and severe gastrointestinal GvHD. The impact of *NOD2/CARD15* mutations was more prominent for HLA-identical sibling transplantations but was

also observed in unrelated donor transplantation. Mutations proved to be independent risk factors for transplant-related mortality. Our findings indicate a major role of monocyte/macrophage dysfunction in the pathophysiology of GvHD and strongly suggest a future risk assessment or even donor selection through *NOD2/CARD15* typing. (Blood. 2004;104:889-894)

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## Introduction

Acute graft-versus-host disease (GvHD) is still a major and the most severe complication following allogeneic stem cell transplantation (SCT). Although activation of donor T lymphocytes by minor or major HLA differences plays a pivotal role in induction of GvHD, there is increasing evidence of involvement of cytokine-mediated inflammation.<sup>1,2</sup> Experimental models indicate the primacy of gastrointestinal damage: conditioning-related damage of the intestinal epithelium results in bacterial translocation followed by increased cytokine release by macrophages/monocytes and T-cell activation.<sup>3</sup> Clinically, gastrointestinal GvHD is the most severe manifestation of acute GvHD, occurs only in a subgroup of patients, and frequently precedes multiorgan complications.

The *NOD2/CARD15* protein has been described as an intracellular sensor to muramyl dipeptide (a compound of the bacterial cell wall) mediating consecutive nuclear factor  $\kappa$  B (NF- $\kappa$ B) activation<sup>4</sup> and belongs to a large family of proteins involved in intracellular pathogen recognition.<sup>5</sup> *NOD2/CARD15* expression is—as known so far—restricted to intestinal epithelial cells (IECs) and cells of monocyte/macrophage lineage.<sup>5,6</sup> A variety of single nucleotide polymorphisms (SNPs) in the *NOD2/CARD15* gene have been reported. Within these SNPs, high-risk alleles in SNPs 8, 12, and 13 have been identified which confer an increased risk for Crohn

disease.<sup>7,8</sup> Patients heterozygous for these mutated forms have a 2- to 4-fold increased risk to develop Crohn disease, and the risk increases to 20- to 40-fold in homozygous patients.<sup>9</sup> Based on the homologies in the pathophysiology of GvHD and inflammatory bowel disease, we speculated that the genetic background shown for *NOD2/CARD15* polymorphisms might also have an impact on the pathophysiology of GvHD.

## Patients, materials, and methods

### Patients

Included in the study were 169 consecutive patients admitted at our transplant unit for allogeneic SCT between June 1998 and July 2003. Conditioning and prophylactic immunosuppression were performed according to standard protocols; reduced-intensity conditioning (RIC) consisted of fludarabine, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), and melphalan (Table 1).<sup>10</sup> There were 78 patients who received grafts from HLA-identical siblings (matched related donors [MRDs]), 87 who received grafts from unrelated donors (URDs), and 4 patients who received grafts from one-HLA-antigen-different relatives (RDs). Within the unrelated donor group, 64 donor/recipient pairs were matched for HLA-A, HLA-B, DRB1, and DQB1 as determined by low-resolution typing for class I and

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**Table 1. Major characteristics of patients, donors, and transplantation procedures in donor/recipient pairs without and with mutated *NOD2/CARD15* polymorphisms**

	Patient/donor pairs without mutations (wild type)	Any patient/donor mutation
<b>Patient characteristics</b>		
Age at transplant, y (range)	45 (16-65)	42 (17-64)
Sex, no. female/no. male	49/70	19/31
<b>Underlying disease</b>		
Acute leukemia, no. (%)	55 (46)	23 (46)
Myeloproliferative disorder, no. (%)	27 (23)	10 (20)
Lymphoma/myeloma (%), no.	37 (31)	17 (34)
<b>Stage at SCT</b>		
Early/intermediate leukemia, no. (%)	67 (56)	28 (56)
Advanced stage, no. (%)	52 (44)	22 (44)
<b>Donor characteristics</b>		
HLA-identical sibling, no. (%)	55 (46)	23 (46)
Matched unrelated donor, no. (%)	61 (51)	26 (52)
DfRelDonor, no. (%)	3 (3)	1 (2)
Sex, no. female/no. male	55/64	17/33
Age at donation, y (range)	41 (16-66)	40 (17-61)
<b>Stem cell source</b>		
Marrow, no. (%)	28 (24)	15 (30)
Peripheral blood, no. (%)	91 (76)	35 (70)
<b>Conditioning regimen</b>		
Standard, no. (%)	70 (59)	31 (60)
RIC, no. (%)	49 (41)	21 (40)
<b>GvHD prophylaxis</b>		
CsA/MTX, no. (%)	73 (61)	32 (64)
CsA/MMF, no. (%)	46 (39)	18 (36)
<b>T-cell depletion</b>		
None, no. (%)	52 (44)	18 (36)
In vivo; eg, ATG, Campath, no. (%)	51 (43)	24 (48)
CD34 selection, no. (%)	16 (13)	8 (16)

Standard conditioning regimens consisted of 8 Gy to 12 Gy fractionated total body irradiation followed by high-dose cyclophosphamide or classic busulfan/cyclophosphamide; reduced-intensity conditioning (RIC) consisted mainly of fludarabine/BCNU and melphalan regimens. For T-cell depletion, serotherapy with antithymocyte globulin (ATG) or, in a few patients, with Campath, was given in the course of pretransplantation conditioning. There were 24 patients who had been enrolled on a protocol for CD34 selection. All criteria were equally distributed between patients without ( $n = 119$ ) and with ( $n = 50$ ) mutations of donor or recipient.

DfRelDonor indicates that the donor is a one-HLA-antigen-different relative; CsA, cyclosporin A; MTX, methotrexate; and MMF, mycophenolate-mofetil.

high-resolution typing for class II, whereas 1- or 2-allele mismatches for DRB1 and DQB1 had been accepted in the remaining 23 pairs. GvHD was graded according to Glucksberg criteria<sup>11</sup> and major outcome variables such as transplant-related mortality (TRM; death in remission) and causes of death were recorded in a database updated monthly. Median observation time for surviving patients or patients dying from relapse was 450 days (range, 33-1767 days); median observation time for patients dying from TRM was 172 days (range, 14-1065 days).

#### Collection of DNA samples and informed consent

DNA from 169 patients and from 168 of their donors was collected, frozen, and retrospectively analyzed to assess the role of *NOD2/CARD15* mutations. DNA from donors and recipients had been prepared from peripheral blood cells at the time of admission for SCT. At admission and prior to stem cell collection, patients and donors had given their informed written consent to prospective collection of DNA samples for assessment of genetic risk factors associated with GvHD and TRM by SNP analysis for cytokines and inflammatory proteins. The collection of samples had been approved by the local ethics committee (institutional review board approval no. 02/220).

#### Allelic discrimination of the *NOD2/CARD15* gene

Single nucleotide polymorphisms 8, 12, and 13 were determined by a Taqman protocol.<sup>12</sup> Control samples that were confirmed by sequencing were included in each Taqman run. The primers and probes were synthesized by MWG Biotech (Ebersberg, Germany). Probes were synthesized with reporter dye 6-FAM or TET covalently linked at the 5' end and a quencher dye TAMRA linked to the 3' end of the probe. The optimized reaction mix of a final volume of 10  $\mu$ L consisted of Universal Master Mix (PE Applied Biosystems, Foster City, CA), 400 nM of each forward and reverse primer, 250 nM of each fluorogenic probe, and the DNA template (20 ng/well). All reactions were performed in a 384-well plate (Abgene, Epsom, United Kingdom) and amplified using the thermocycler Primus-HT (MWG Biotech). The cycle conditions were 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds (melting step) and 60°C for 1 minute (anneal/extend step). After the polymerase chain reaction (PCR) run, the released fluorescence was measured by the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems).

Increases in the amount of reporter dye fluorescence during the 40 cycles of amplification were monitored using Sequence Detector software (SDS version 1.6; PE Applied Biosystems). Changes in reporter dye fluorescence of 6-FAM versus changes in reporter dye fluorescence of TET were plotted (homozygous FAM versus homozygous TET versus heterozygous FAM/TET).

#### Statistical considerations regarding sample size

At the time of availability of the *NOD2/CARD15* assay for our patient population, we had collected DNA from 169 consecutive patients and their respective donors with an actual TRM of 33% observed in these patients. Based on the distribution of unmutated and mutated *NOD2/CARD15* SNPs in the control population reported by Hampe et al,<sup>12</sup> we assumed a 2:1 ratio of patients with wild-type or mutated SNPs. We further assumed that occurrence of *NOD2/CARD15* mutations should at least double the incidence of TRM from 25% to 50% or result in a hazard ratio of 2.0 between patients with wild-type SNPs and those with mutated SNPs. Given a doubling of TRM, we calculated that the available sample size of 169 would be sufficient to detect a difference concerning TRM with a significance of  $P$  equal to .05 and a power of 84%. Given a hazard ratio of 2.0, the difference would be detected with a power of 72%. Calculations were performed using the respective models of the PASS software (version 2000; NCSS, Kaysville, UT).

#### Clinical and statistical analysis

Three major outcome variables (TRM at 365 days is equal to 1-year TRM; overall grade of severe acute GvHD, grade III/IV; and stage of gastrointestinal GvHD) were analyzed in relation to *NOD2/CARD15* mutations. Cumulative incidence reflecting the competing risks of death from other toxicities or relapse for GvHD, and death from relapse for TRM were calculated for each of these parameters. Time to the clinical event (TRM, GvHD) was measured from the date of SCT. For overall GvHD and gastrointestinal GvHD, events occurring until day 100 were included in the model. For 1-year TRM, events occurring until day 365 after SCT were included in the model. Stepwise multivariate Cox regression models were adjusted testing the independent prognostic relevance of *NOD2/CARD15* mutations. The limit for reverse selection procedures was 0.2. For the multivariate comparison of major risk factors for TRM and GvHD, only age of the patient, disease stage at the time of transplantation, type of donor (HLA-identical sibling vs URD vs RD), and *NOD2/CARD15* mutation status were considered. For analysis of *NOD2/CARD15* SNPs, the less frequent high-risk alleles associated with Crohn disease and an impaired NF- $\kappa$ B production were defined as mutated, whereas the more frequently observed alleles were referred to as wild-type alleles. For analysis of mutations in relation to clinical events, groups were formed according to occurrence of (1) any mutations in recipients or donors and (2) any mutations in recipients only, donors only, or in both.

Cumulative incidence allowing adjustment for competing risks was calculated using NCSS software (version 2004), further statistical analysis was performed by the help of SPSS software (version 11.05, Chicago, IL).

## Results

### Frequency of mutated NOD2/CARD15 alleles

NOD2/CARD15 mutations occurred with a frequency of 21.8% in patients and 13.7% in donors typed in this study. A homozygous mutation was observed in only one patient and in his HLA-identical donor, whereas all other patients and donors with mutations were heterozygous. Calculated haplotype frequencies for mutated SNP 8 were 0.056 for recipients (R) and 0.045 for donors (D); for mutated SNP 12 were 0.021 (R) and 0.009 (D); and for mutated SNP 13 were 0.027 (R) and 0.034 (D). Thus, the haplotype frequencies were close to the range of controls reported in studies on Crohn disease<sup>12</sup> (SNP 8: 0.0478; SNP 12: 0.0072; and SNP 13: 0.0406). This resulted in an overall percentage of 70.5% of transplantations where both donors and recipients had unmutated SNPs (wild-type group). In 22 pairs (13%), only recipients had mutated SNPs (R mut); in 16 pairs (9.5%), mutations occurred in donors only (D mut); and in 12 pairs (7%), both donors and recipients revealed mutations (R & D mut). In our consecutive cohort of patients, patient- and donor-specific characteristics as well as transplant-specific procedures were equally distributed between patient/donor pairs with and without mutations (Table 1).

### Outcome in relation to occurrence of NOD2/CARD15 mutations in donor or recipients

Patients were followed for a median of 16 months (range, 0.5 to 59 months). We first compared the outcome of patients from wild-type recipient/donor pairs (n = 119) with the outcome of patients in pairs with any type of mutations (n = 50). Overall, severe GvHD grade III/IV, severe gastrointestinal GvHD, and TRM at one year were significantly increased in pairs with NOD2/CARD15 mutations as compared with the wild-type group (Table 2). Deaths from relapse occurred in both groups: 18 of 119 patients with wild-type SNPs and 7 of 50 patients with mutated pairs (data not shown).

When mutations were split according to their occurrence in recipients or donors or both (Table 3; Figure 1), there was an increased risk of overall severe GvHD in recipients with donor mutations and a dramatic rise for both overall and gastrointestinal GvHD in the small subgroup of pairs with mutations of both recipients and donors. As a consequence, cumulative incidence of 1-year TRM increased from 20% in wild-type recipient/donor pairs to 49% in pairs with recipient (P = .03), to 59% in pairs with donor

**Table 3. Detailed analysis of cumulative incidence for GvHD grade III/IV, gastrointestinal GvHD stage II/IV, and TRM at one year in relation to donor and recipient NOD2/CARD15 mutations**

	Cumulative incidence, %	95% CI for cumulative incidence, %	P	Hazard ratio	95% CI for hazard ratio
<b>GvHD III/IV</b>					
Wild type	19	13-27		1.0	
R pos	36	21-61	.07	2.1	(0.9-4.7)
D pos	44	25-76	.02	2.8	(1.2-6.5)
R&D pos	58	36-94	.002	3.9	(1.7-9.2)
<b>GI GvHD</b>					
Wild type	18	12-27		1.0	
R pos	36	21-63	.07	2.1	(0.9-4.8)
D pos	31	15-65	.16	2.0	(0.8-5.3)
R&D pos	56	36-94	.001	4.3	(1.8-10.1)
<b>1-year TRM</b>					
Wild type	20	14-29		1.0	
R pos	49	31-76	.03	2.2	(1.1-4.6)
D pos	59	38-91	.004	3.1	(1.4-6.5)
R&D pos	83	65-100	.001	3.9	(1.7-8.6)

Wild type indicates donor and recipient unmutated (n = 119); R pos, mutations in recipients only (n = 22); D pos, mutations in donors only (n = 16); R&D pos, mutations of both recipient and donor (n = 12). Comparison between groups was performed by Cox regression analysis. For each group, significance and hazard ratios are given.

(P = .004), and to 83% if donor and recipient revealed mutations (P = .001). Again, deaths from relapses were almost equally distributed within these subgroups (data not shown).

The strong association of TRM with NOD2/CARD15 mutations was partially explained by an increased risk not only for death from GvHD but also from respiratory failure due to diffuse primary or secondary pulmonary damage. Whereas in the wild-type group only 11 of 30 (37%) deaths in remission were caused by GvHD and respiratory failure, the proportion increased to 17 of 26 (65%) in pairs with NOD2/CARD15 mutations.

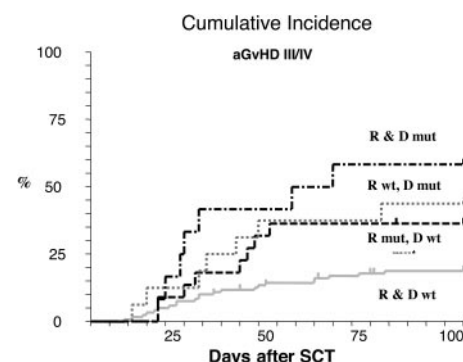
### Relevance of observations for HLA-identical sibling versus unrelated donor transplantations and multivariate analysis of risk factors associated with TRM

We next compared the relevance of donor and recipient mutations in the different immunogenetic subgroups included in this analysis. Although the associations of TRM with NOD2/CARD15 mutations

**Table 2. Univariate analysis of cumulative incidence of GvHD and TRM**

	Cumulative incidence, %	95% CI for cumulative incidence, %	P	Hazard ratio	95% CI for hazard ratio
<b>GvHD III/IV</b>					
Wild type	19	13-27	.001	1.0	
Mutated	44	32-60		2.7	(1.5-4.9)
<b>GI GvHD</b>					
Wild type	18	12-27	.004	1.0	
Mutated	40	28-56		2.5	(1.4-4.7)
<b>1-year TRM</b>					
Wild type	20	14-29	.000	1.0	
Mutated	58	45-75		2.8	(1.7-4.9)

The table shows univariate analysis of cumulative incidence of acute GvHD grade III/IV, gastrointestinal (GI) GvHD stage II/IV, and TRM at one year in patient/donor pairs with unmutated NOD2/CARD15 SNPs (wild type, n = 119) versus pairs with either recipient or donor mutations (mutated, n = 50). Cox regression was used to compare groups and analyze hazard ratios. CI indicates confidence interval.



**Figure 1. Cumulative incidence of severe acute GvHD (grade III/IV) in relation to NOD2/CARD15 SNPs.** R indicates recipient; D: donor; R&D wt: unmutated (wild-type) SNPs in both R and D (n = 119); R mut/D wt: mutated SNPs in recipients only (n = 22); R wt/D mut: mutated SNPs in donors only (n = 16); R&D mut: mutated SNPs in donors and recipients (n = 12). Overall, differences were highly significant: P = .001 for the whole group; P = .07 for R mut D wt; P = .004 for R wt/D mut, and P = .001 for R&D mut if compared with unmutated R&D.

**Table 4. Univariate analysis of cumulative incidence of TRM at one year**

	Cumulative incidence, %	95% CI for cumulative incidence, %	P	Hazard ratio	95% CI for hazard ratio
<b>HLA-identical sibling</b>					
Wild type (55)	14	18-42	NA	1.0	
R pos (9)	74	49-100	.000	7.9	2.6-24.0
D pos (6)	58	27-100	.022	4.9	1.3-18.9
R & D pos (8)	75	50-100	.004	6.4	1.8-23.0
<b>Matched unrelated donor</b>					
Wild type (61)	28	18-42	NA	1.0	
R pos (13)	34	14-73	NS	1.1	0.4-3.3
D pos (9)	56	31-100	NS	2.4	0.9-6.5
R & D pos (4)	75	43-100	.01	4.2	1.4-12.5

Univariate analysis of cumulative incidence of TRM at one year in patient/donor pairs with unmutated *NOD2/CARD15* SNPs (wild type) versus pairs with either recipient (R pos) or donor mutations (D pos) or mutations in both (R & D pos). Separate analyses were performed for HLA-identical sibling transplantation and matched unrelated donor transplantations. Comparison between groups was performed using log-rank analysis.

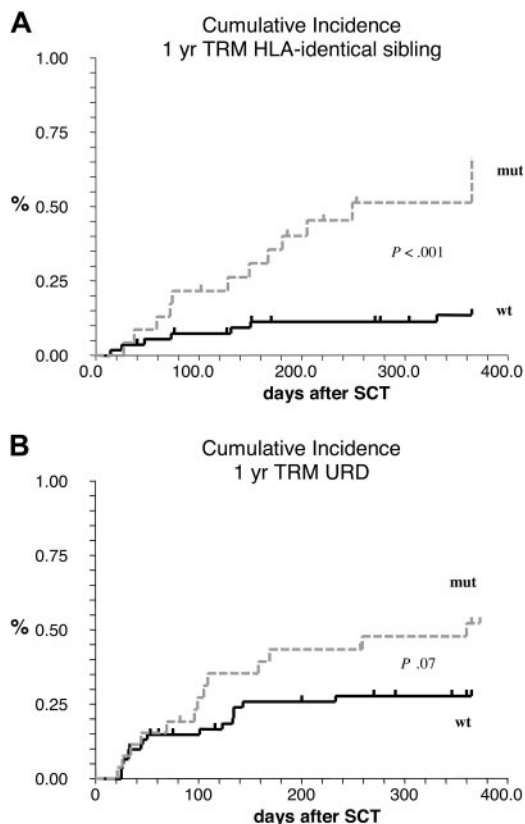
CI indicates confidence interval; NA, not applicable; and NS, not significant.

\*Overall *P* value.

were more prominent for HLA-identical sibling transplantations, there were parallel trends for URD transplantations (Table 4; Figure 2). Within the URD group, cumulative incidence of 1-year TRM rose from 27% in wild-type pairs to 55% in mutated pairs, if recipients and donors were matched for HLA-A, HLA-B, DRB1, and DQB1. TRM increased from 29% to 50% in pairs with one or 2 DRB1- or DQB1-allele mismatches (data not shown). Studies in larger cohorts of unrelated donor transplantations are clearly needed to assess the definitive role of *NOD2/CARD15* mutations in unrelated donor transplantation and in relation to the class I and

class II allele or even antigen mismatches. The small group of antigen mismatched-related transplantations showed a high mortality and did not allow any assessment of the role of *NOD2/CARD15* mutations in this particular subgroup.

In multivariate Cox regression analyses, age, donor type, and *NOD2/CARD15* mutations in donors alone or in both recipients and donors were independent risk factors for TRM (Table 5). The hazard ratios for TRM following allogeneic SCT concerning *NOD2/CARD15* mutations were 2.4 ( $P = .02$ ; 95% confidence interval [CI] 1.1-5.0) for recipient mutations, 2.5 ( $P = .02$ ; 95% CI 1.2-5.4) for donor mutations, and 6.0 ( $P = .000$ ; 95% CI 2.6-14.1) in the case of simultaneous mutations in donors and recipients. When we included overall GvHD grade II-IV as a risk factor for TRM in our model, GvHD ( $P = .006$ ) as well as *NOD2/CARD15* mutations ( $P = .000$ ) remained significant risk factors for TRM. Even after inclusion of severe GvHD grade III/IV, the contribution of *NOD2/CARD15* remained significant (data not shown), clearly confirming the strong and independent impact of *NOD2/CARD15* mutations on the outcome of allogeneic SCT.



**Figure 2. Cumulative incidence of 1-year transplant-related mortality (TRM) in relation to *NOD2/CARD15* SNPs.** Wt indicates unmutated SNPs (wild-type) in both recipients and donors; mut: any mutated SNP in donor or recipient or in both. (A) HLA-identical sibling transplantations; wt: n = 55, mut: n = 23. (B) HLA-matched unrelated donor transplantations; wt: n = 61, mut: n = 25.

**Table 5. Multivariate risk factor analysis for 1-year TRM**

	No.	No. events, 1-year TRM	Hazard ratio	95% CI	P
<b>Age</b>					
≤ 40 y	58	15	1.0		
> 40 y	111	38	2.2	1.1-4.0	.02
<b>Stage at transplantation</b>					
Early/intermediate	95	27	1.0		
Advanced	74	26	1.3	0.7-2.2	NS
<b>Donor type</b>					
HLA-identical sibling	78	20	1.0		
Unrelated	87	29	1.7	0.9-3.2	NS
HLA-DfRel	4	4	10.7	3.4-33.3	.000
<b><i>NOD2/CARD15</i></b>					
Wild type	119	26	1.0		
R pos	22	10	2.4	1.1-5.0	.02
D pos	16	9	2.5	1.2-5.4	.02
R&D pos	12	8	6.0	2.6-14.1	.001

Relevant patient and donor pretransplantation characteristics were compared with *NOD2/CARD15* mutations in recipients (R pos), donors (D pos), and both recipients and donors (R&D pos).

HLA-DfRel indicates that the donor is a one-HLA-antigen-different relative.

\*Overall, or global, *P* value.

## Discussion

Our study is the first to report a strong association of mutations in SNPs of the *NOD2/CARD15* gene with severe GvHD, gastrointestinal GvHD, and TRM following allogeneic SCT. So far, only one casuistic report on de novo occurrence of Crohn disease after allogeneic SCT suggested that donor mutations might transfer the risk of Crohn disease to the patients and result in an increased rate of complications.<sup>13</sup> Our findings, however, indicate a major direct impact of *NOD2/CARD15* mutations in both recipient and donor cells on outcome following allogeneic SCT from related as well as unrelated donors. Although the exact contribution of each single SNP mutation and, more importantly, the various rare combinations of donor and recipient mutations can only be defined after analysis of large cohorts of patients, our data allow identification of patient/donor pairs with an increased risk of GvHD and subsequent death from TRM. Thus our findings should have major implications for pathophysiologic concepts of GvHD as well as the clinical management of patients.

As well known for toll-like receptors (TLRs) on the cell membrane, *NOD2/CARD15* proteins are reported to play a major role in the intracytoplasmatic response to bacterial cell wall products (muramyl dipeptide) and subsequent NF- $\kappa$ B activation in monocytes/macrophages as well as in specialized epithelial cells of the ileum.<sup>4,14,15</sup> A contribution of an altered epithelial reactivity in the ileum is not unexpected in GvHD, and an impaired antimicrobial response with diminished defensin production<sup>15</sup> and increased translocation of bacteria as shown in experimental models<sup>16</sup> could well contribute to excess morbidity in pairs with mutations of the recipient or in both recipient and donors. The additional and strong donor associations are surprising for several reasons. Recipient monocytes are rapidly replaced by donor cells in allogeneic SCT, and our data indicate a broader role of donor monocytes and their reactivity to bacterial toxins in GvHD. So far, the interaction of bacterial toxins and monocytes/macrophages in GvHD has been explained by up-regulation of inflammatory cytokine responses in IFN $\gamma$ -sensitized macrophages.<sup>17-19</sup> The association of *NOD2/CARD15* mutations characterized by a diminished NF- $\kappa$ B response, however, with GvHD and TRM suggest a predominant role of an impaired monocyte/macrophage response resulting in dysregulation of inflammation. The exact mechanisms of interaction of a diminished monocyte/macrophage response and altered behavior of epithelial cells resulting in a possible defect in the defense against bacteria, and T-cell activation in allogeneic SCT remains to be elucidated. Our findings, however, strongly remind us of van Bekkum and Knoan's results,<sup>20</sup> which suggested that translocation of intestinal microflora directly interferes with T-cell activation and donor T-cell expansion. The predominant role of the gastrointestinal tract in T-cell activation is further supported by a recent study

showing that Peyer patches<sup>21</sup> are essential for induction of T-cell responses in murine GvHD.

Besides Crohn disease and other inflammatory bowel diseases, the role of an impaired monocyte/macrophage response due to *NOD2/CARD15* mutations has so far only been discussed in the context of spondylarthritis<sup>22,23</sup> and other immune-mediated diseases like psoriatic arthritis and lupus erythematoses.<sup>24,25</sup> Our data, however, suggest not only an increased mortality from GvHD in patients bearing *NOD2/CARD15* mutations but also from respiratory failure. Thus, it might well turn out, that, in addition to gastrointestinal inflammation, monocyte/macrophage dysfunction in organs like the lung is affected by *NOD2/CARD15* function and polymorphisms thus contributing to pathophysiology of pulmonary complications. In allogeneic SCT, a gut-liver-lung axis<sup>1</sup> of impaired elimination of intestinal lipopolysaccharide (LPS) by the liver has been postulated to be involved in development of pulmonary damage. Direct involvement of an impaired monocyte/macrophage or even epithelial response in the lung, however, might be an alternative explanation and should be addressed in careful analyses in future studies.

Clinically, our data suggest that avoiding a stem cell graft from a donor bearing mutations, at least if the patient has mutated SNPs, might reduce the risk of developing severe complications. This is also strongly supported by the results of multivariate analysis. As *NOD2/CARD15* mutations are not linked to HLA and the associations described in our study turned out to be true for both related and unrelated donors, our results might have a future influence on typing and donor selection strategies. Of course, assessment of *NOD2/CARD15* mutations in further cohorts of donor/recipients in both related and especially unrelated SCT is required to verify this hypothesis. Our data strongly argue for a detailed analysis comparing the relevance of allele matching with assessment of *NOD2/CARD15* mutations in unrelated donor transplantation.

In summary, our data describe *NOD2/CARD15* mutations as a new and highly significant genetic factor allowing risk assessment in a subgroup of patients receiving allogeneic SCT. As discussed for Crohn disease, consideration of GvHD as a result of a monocyte/macrophage defect resulting in impaired tolerance against bacteria rather than of uncontrolled excess stimulation should widen pathophysiologic and hopefully also prophylactic and therapeutic concepts of this major complication of allogeneic SCT.

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## References

- Cooke KR, Olkiewicz K, Erickson N, Ferrara JL. The role of endotoxin and the innate immune response in the pathophysiology of acute graft versus host disease. *J Endotoxin Res.* 2002;8:441-448.
- Holler E. Cytokines, viruses, and graft-versus-host disease. *Curr Opin Hematol.* 2002;9:479-484.
- Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood.* 2000;95:2754-2759.
- 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.
- Inohara N, Ogura Y, Nunez G. Nods: a family of cytosolic proteins that regulate the host response to pathogens. *Curr Opin Microbiol.* 2002;5:76-80.
- Berrebi D, Maudinas R, Hugot JP, et al. Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. *Gut.* 2003;52:840-846.
- Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature.* 2001;411:599-603.
- Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature.* 2001;411:603-606.
- Bonen DK, Cho JH. The genetics of inflammatory bowel disease. *Gastroenterology.* 2003;124:521-536.

10. Wasch R, Reisser S, Hahn J, et al. Rapid achievement of complete donor chimerism and low regimen-related toxicity after reduced conditioning with fludarabine, carmustine, melphalan and allogeneic transplantation. *Bone Marrow Transplant.* 2000;26:243-250.
11. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation.* 1974;18:295-304.
12. Hampe J, Grebe J, Nikolaus S, et al. Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet.* 2002;359:1661-1665.
13. Sonwalkar SA, James RM, Ahmad T, et al. Fulminant Crohn's colitis after allogeneic stem cell transplantation. *Gut.* 2003;52:1518-1521.
14. Bonen DK, Ogura Y, Nicolae DL, et al. Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. *Gastroenterology.* 2003;124:140-146.
15. Ogura Y, Lala S, Xin W, et al. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut.* 2003;52:1591-1597.
16. Hisamatsu T, Suzuki M, Reinecker HC, et al. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology.* 2003;124:993-1000.
17. Cooke KR, Hill GR, Crawford JM, et al. Tumor necrosis factor-alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J Clin Invest.* 1998;102:1882-1891.
18. Lampert IA, Moore RH, Huby R, Cohen J. Observations on the role of endotoxin in graft-versus-host disease. *Prog Clin Biol Res.* 1988;272:351-359.
19. Cooke KR, Gerbitz A, Crawford JM, et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. *J Clin Invest.* 2001;107:1581-1589.
20. van Bekkum DW, Knaan S. Role of bacterial microflora in the development of intestinal lesion from graft-versus-host reactions. *J Natl Cancer Inst.* 1977;58:787-790.
21. Murai M, Yoneyama H, Ezaki T, et al. Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. *Nat Immunol.* 2003;4:154-160.
22. Crane AM, Bradbury L, Van Heel DA, et al. Role of NOD2 variants in spondylarthritis. *Arthritis Rheum.* 2002;46:1629-1633.
23. Breban M, Said-Nahal R, Hugot JP, Miceli-Richard C. Familial and genetic aspects of spondyloarthropathy. *Rheum Dis Clin North Am.* 2003;29:575-594.
24. Ferreira-Vidal I, Garcia-Mejide J, Carreira P, et al. The three most common CARD15 mutations associated with Crohn's disease and the chromosome 16 susceptibility locus for systemic lupus erythematosus. *Rheumatology (Oxford).* 2003;42:570-574.
25. Rahman P, Bartlett S, Siannis F, et al. CARD15: a pleiotropic autoimmune gene that confers susceptibility to psoriatic arthritis. *Am J Hum Genet.* 2003;73:677-681.