Blood First Edition Paper, prepublished online February 22, 2010; DOI 10.1182/blood-2009-09-243840

INSUFFICIENT EVIDENCE FOR ASSOCIATION OF NOD2/CARD15 OR OTHER INFLAMMATORY BOWEL DISEASE-ASSOCIATED MARKERS ON GVHD INCIDENCE OR OTHER ADVERSE OUTCOMES IN T-REPLETE, UNRELATED DONOR TRANSPLANTATION

Yume Nguyen[1], Abed Al-Lehibi[1], Elizabeth Gorbe[1], Ellen Li[1], Michael Haagenson[3], Tao Wang[4], Stephen Spellman[2], Stephanie J. Lee[5] and Nicholas O. Davidson[1,6]

[1]Division of Gastroenterology, Washington University School of Medicine, St. Louis, MO; [2] National Marrow Donor Program, Minneapolis, MN; [3] Center for International Blood & Marrow Transplant Research, Minneapolis, MN; [4] Division of Biostatistics, Medical College of Wisconsin, Milwaukee, WI; [5] Fred Hutchinson Cancer Research Center, Seattle, WA

[6]To whom communication should be addressed:
Nicholas O. Davidson, MD
Division of Gastroenterology
Washington University School of Medicine
660 S. Euclid Ave, Campus Box 8124
St. Louis, MO 63110

email: nod@wustl.edu

Phone: (314) 362-2027

Key words: IBD susceptibility alleles, Graft versus host disease, Predictive testing, NOD2/CARD15, IRGM, IL-23R

Running title: No evidence for IBD susceptibility markers in GVHD

ABSTRACT

Previous European studies suggest NOD2/CARD15 and IL-23R donor or recipient variants are associated with adverse clinical outcomes in allogeneic hematopoietic stem cell transplantation. We re-examined these findings as well as the role of another inflammatory bowel disease (IBD) susceptibility gene (IRGM) on transplant outcomes in 390 US patients and their matched unrelated donors, accrued between 1995-2004. Patients received T-replete grafts using mostly myeloablative conditioning regimens. Multivariate analyses were performed for overall survival, disease-free survival, transplant-related mortality, relapse, and acute and chronic graft-versus-host disease (GVHD). Of 390 pairs, NOD2/CARD15 variant single nucleotide polymorphisms (SNPs) were found in 14% of donors and 17% of recipients. In 3% both donor and recipient had a mutant SNP. 13% of donors and 16% of recipients had variant IL23R SNPs, with 3% having both donor and recipient variants. 23% of both donors and recipients had variant IRGM SNPs. None of the 3 IBD-associated alleles showed a statistically significant association with any adverse clinical outcomes. Our results do not support an association between the 3 IBD-associated SNPs and adverse outcomes following matched unrelated donor hematopoietic cell transplantations in US patients.

INTRODUCTION

Allogeneic hematopoietic stem cell transplant (HSCT) is an important and potentially curative intervention in the management of a range of hematologic malignancies, but the efficacy and outcome of this treatment is frequently limited by its inherent immunologic sequelae. Among the dominant complications of HSCT, graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality, and considerable attention has focused on strategies to limit its development while not completely eliminating the beneficial graft-versus-leukemia effect [1]. These strategies have included allelic HLA-matching, appropriate donor selection, T-cell depletion, and other prophylactic immunosuppressive regimens.

Earlier observations revealed extensive gastrointestinal mucosal damage in patients with GVHD, raising the suggestion that resultant bacterial translocation might play a role in both the initiation and maintenance of a systemic inflammatory response as a result of the conditioning regimen and which could potentially represent a key inciting event in the pathogenesis of GVHD [2]. These observations, coupled with an extensive literature pointing to the role of bacterial flora in the etiology of idiopathic inflammatory bowel disease (IBD), have together reinforced the concept that luminal bacterial communities may represent key elements in the host response to injury [3]. Indeed, genome-wide association studies have identified more than 30 distinct loci linked with susceptibility to Crohn's disease including genes involved in innate host defense[4, 5 6 7]. Accordingly, an emerging consensus suggests that GVHD and IBD, both immune-mediated inflammatory conditions, may share overlapping pathways in their pathogenesis.

Among the many candidate IBD-associated genes, NOD2/CARD15 was initially identified as a susceptibility gene in Crohn's disease with a frequency of 7.7%-9.1% in the healthy U.S. population [8]. NOD2/CARD15 encodes a cytosolic protein that recognizes a bacterial cell wall component, muramyl dipeptide, and results in downstream activation of innate pathways of host defense. Three informative single nucleotide polymorphisms (SNPs) in NOD2/CARD15 have been identified including Leu1007fsinsC, a cytosine insertion that produces a 33 amino acid protein truncation, and two missense mutations Arg702Trp and Gly908Arg (SNPs 8, 12 and 13, respectively)[9, 10].

The importance of these allelic variants in the pathogenesis of GVHD was suggested by Holler et al, who initially demonstrated that SNPs 8, 12, and 13 of the NOD2/CARD15 gene in either donor or recipient (from a European cohort) was associated with increased incidence and severity of acute GVHD, increased transplant-related mortality, and reduced survival in HLA-matched sibling transplants. These effects were most pronounced in pairs with the risk variant in both donor and recipient [11]. A follow-up study by the same group demonstrated an increased risk of severe acute GvHD, but not mortality in unrelated donor (URD) transplants. In addition, when broken down by individual polymorphism, SNP 13 was the only significant risk factor [12]. While these findings suggested the importance of NOD2/CARD15 variants in GVHD, other studies have demonstrated conflicting results that failed to confirm these associations [13-15].

Accordingly, the aim of the current study was to evaluate the impact of NOD2/CARD15 and two other IBD-associated SNPs, on the outcomes of HSCT in a U.S. population. We selected IRGM (immunity-related GTPase family, M) a gene product involved in autophagy and intracellular pathogen clearance, since variations in or near the IRGM locus have been associated with increased susceptibility to Crohn's Disease [16], and IL23R (interleukin 23 receptor) since variants appear to have a protective role in the development of IBD [17]. In addition, recent findings in adults[18] and, more recently, in children[19]indicated a protective effect of the variant IL23R in donors from the development of GVHD after HSCT. Nevertheless, our findings, fail to show a significant effect (at the $p<0.01$ level) of variants in these SNPs and the outcomes of HSCT.

MATERIALS AND METHODS

Patient populations and treatment regimen

Pre-transplant samples from 390 patients and their 10/10 HLA-A, B, C, DRB1 and DQB1-matched unrelated donors from 85 different centers within the United States were provided by the National Marrow Donor Program Research Repository (NMDP, Minneapolis, MN). Patients received T-replete transplants for early stage AML, ALL, CML, MDS accrued between 1995-2004. The vast majority (98%) underwent a myeloablative conditioning regimen (Table 1). GVHD prophylaxis employed combinations of cyclosporine, tacrolimus and methotrexate as detailed in Table 1. Gut decontamination was not routinely performed, but the prevalence of this practice varies widely and was not systematically recorded in the data collected. Patients and donors were of predominately European American descent, 92.2% and 94.5%, respectively with 6.7% racial/ethnic minorities (Table 1). Median time of follow-up was 77 months (range 12-149). Approval was obtained from the Washington University Human Studies Committee, and all patients and donors gave their informed and written consent to the NMDP for genetic analysis of their biological samples in accordance with the Declaration of Helsinki.

Genotyping of SNPS

DNA was extracted from frozen whole blood samples collected from donors and patients pre-transplant using QIAGEN Blood Kit with Proteinase K. Genomic DNA was isolated using a QIAmp DNA Blood Mini Kit (QIAgen, Germany) following the manufacturer's instructions. Control samples of each genotype, which were confirmed by sequencing, were included in each run. DNA was genotyped for three NOD2/CARD15 SNPs (rs2066844, rs2066845 and rs2066847), two IL23R SNPs (rs11209026 and rs11465804), and two IRGM SNPs (rs4958847 and rs13361189) using Sequenom and/or Taqman SNP Genotyping Assay (PE Applied Biosystems, Foster City, CA, USA). Each reaction was performed in a 96-well plate. The optimized reaction mix of a final volume of 50 µL consisted of Universal Master Mix and the DNA template. The sequence of the primers used is available upon request.

Data Analysis and Statistical Considerations

Individuals heterozygous for at least one of the three NOD2/CARD15 SNPs analyzed (SNPs 8, 12, and 13) were considered *variant*, whereas individuals lacking these SNPs were considered *wild-type*. Clinical variables analyzed include CMV status, disease, graft source, GVHD prophylaxis, Karnofsky score, donor and patient age, donor/recipient gender mismatch, myeloablative vs. non-myeloablative conditioning, and year of transplant. Diagnosis for acute or chronic GVHD was based on clinical signs, and/or histopathological findings in biopsies from skin, liver and the gastrointestinal tract. Grading of acute GVHD was performed based on the Glucksberg criteria [20]. Multivariate analyses were performed using Cox proportional hazards regression analysis for overall (OS) and disease free survival (DFS), transplant-related mortality (TRM), relapse, and acute and chronic GVHD, adjusting for the previously mentioned clinical variables. Due to the multiple testing performed within the multivariate analysis, a p-

value of less than 0.01 is considered significant. SAS software version 9.1 (SAS Institute, Cary, NC) was used in all analyses.

RESULTS

Patient characteristics

The distribution by age, gender and disease is shown in Table 1. Most (74%) patients receiving transplants were between the ages of 20 and 50, with the majority (63%) being performed for CML. The majority of patients underwent bone marrow grafting (81%) and 98% of subjects underwent a myeloablative conditioning regimen prior to transplant (Table 1).

Frequency distribution of NOD2/CARD15, IL-23R, and IRGM variants

NOD2/CARD15 variant SNPs were found in 56 donors (14%) and 67 recipients (17%), with an overall frequency of 16% in this cohort (Figure 1). In 44 pairs (11%) the mutation was detected in the donor only, in 55 pairs (14%) the mutation occurred in the recipient only, and in 12 pairs (3%), both donor and recipient had a mutant SNP. The small number of donor/recipient pairs that both had a variant NOD2/CARD15 SNP precludes further analysis to determine statistical significance. Frequencies for individual mutated SNPs were 3% for donors and 3% for recipients for SNP 8; 5% of donors and 5% of recipients for SNP 12, and 6.4% for donors and 9.5% for recipients for SNP 13 (Table 2).

For IL23R, 46 (13%) of donors and 55 (16%) of recipients had variant SNPs (n=353 pairs analyzed, Figure 1). In 35 pairs (10%) the mutation occurred in the donor only, in 44 pairs (12%) the mutation occurred in the recipient only, and in 11 pairs (3%) both donor and recipient had variant SNPs (Table 2).

For IRGM, variant SNPs were seen in 90 (23%) of donors and 90 (23%) of recipients (n=385 pairs analyzed, Figure 1). In 66 pairs (17%) the mutation occurred in the donor only, in 66 pairs (17%) the mutation occurred in the recipient only, and in 24 pairs (6%) both donor and recipient had a mutant SNP, (Table 2).

Incidence of clinical endpoints

Table 3 shows the overall incidence of the endpoints which were analyzed. The overall incidence of 100-day severe acute GVHD (grade III-IV) was 21% (17-25%) and 1-year chronic GVHD was 53% (48-58%). GI GVHD rates at 100 days for stages 2-4 was 14% (11-18%) and for stages 3-4 was 8% (5-11%). These rates and those of other major outcomes were similar to previously published results in a US cohort [21]. In comparison, the overall incidence of severe acute GVHD (grade III-IV) for matched unrelated donor transplants cited in the Holler et al study was higher at 28%[12]. Rates of GI GVHD (grade II-IV) were calculated to be higher in the mixed cohort of unrelated and related donor transplants in the Holler et al study (41 of 169 donor/patient pairs or 24.5%)[11]. In general, the incidence of chronic GVHD in European centers has been reported at 27%-57% [22, 23].

None of the 3 IBD-associated alleles showed evidence of a statistically significant association with any of the clinical outcomes at p <0.01 [Figure 2]. In particular there was no association between the three NOD2/CARD15 SNPs (8, 12, or 13) and the incidence of acute or chronic GVHD, or transplant related mortality [Tables 4,5]. For pairs with any variant NOD2/CARD15 SNP the relative risk (RR) of developing acute GVHD (grade II-IV) was 0.99 ([0.68, 1.42], *P*=0.93), severe acute (grade III-IV) GVHD was 0.79 (95% CI=[0.43,1.46], *P*= 0.45), chronic GVHD was 1.02 ([0.70,1.48], *P*= 0.93), and transplant-related mortality was 0.86 ([0.53,1.39], *P*=0.54). Extending this analysis to include acute GVHD grades II-IV, the data demonstrate a RR in pairs with any variant IL23R SNP was 0.74 ([0.49,1.13], *P*=0.16), severe acute GVHD was 0.66 ([0.33,1.34], *P*=0.25), chronic GVHD was 0.84 ([0.56,1.25], *P*=0.39), and TRM was 1.24 ([0.78,1.98], *P*=0.36). For pairs with any variant IRGM SNP the RR of developing acute GVHD (grade II-IV) was 0.97 ([0.70, 1.34], *P*=0.85), severe acute GVHD was 0.82 ([0.47,1.42], *P*= 0.48), chronic GVHD was 1.14 ([0.83,1.56], *P*=0.42), and TRM was 0.59 ([0.37,0.95], *P*=0.03). Even combining all 3 IBD-associated alleles into one "combination variable" (ie where any mutation in any one of the 3 alleles would be considered variant) failed to demonstrate an association with outcome.

DISCUSSION

Among the target organs for GVHD, tissue injury is most pronounced throughout the gastrointestinal tract, which functions as a reservoir for bacterial communities. These resident bacterial flora interact with the host to generate luminal bacterial products, such as lipopolysaccharide, that then translocate across the bowel and perpetuate both a local and systemic inflammatory response[2]. The role of luminal bacterial contents and host interactions in mediating intestinal injury is further underscored by findings in both animal models as well as earlier clinical studies which each suggested that gut decontamination was associated with decreased incidence of GVHD [13, 24, 25]. The further demonstration that polymorphic variants (including NOD2/CARD15) known to modulate the innate immune response may be informative predictors of adverse outcomes in GVHD prompted us to re-examine this association in a US population with standardized transplant protocols. This was an important objective in view of distinctive practice patterns including the use of T-cell depletion, conditioning regimen, gut decontamination, and HLA-matching, that make those earlier findings from European centers difficult to extrapolate to a US population. Gut decontamination, for example, is not routine practice in the U.S., but its use does vary widely amongst various transplant centers. Our findings, by contrast with those earlier reports, suggest that NOD2/CARD15 variants in either the donor or recipient are not significantly associated with clinical outcomes following HSCT in matched unrelated donor transplants. We also concluded that IL-23R donor genotype, despite previous reports of a protective effect on the occurrence of acute GVHD [19]and improved death rates in remission[18], had no effect on outcome. We also included another IBD associated genes, IRGM, which has heretofore not been reported in the context of HSCT outcomes. This gene also did not show an association with any clinically significant outcome. These findings bear further discussion in light of conflicting reports involving the association of NOD2/CARD15 and IL-23R SNPs and HSCT outcomes in different populations.

The initial findings of Holler et al suggested a pathogenic role of NOD2/CARD15 variants in the outcomes of HSCT and were based on observations in 169 recipients and their matched donors from two European centers[11]. Those authors reported that NOD2/CARD15 mutations in either the donor or recipient increased the incidence of transplant related mortality and acute GVHD. Furthermore, pairs where NOD2/CARD15 risk alleles were present in both donor and recipient experienced a 1-year TRM of 83% compared to 20% in wild-type pairs ($P<0.001$). Our study, a retrospective cohort, did not contain sufficient numbers of pairs in which both the donor and recipient had a NOD2/CARD15 mutation and was thus underpowered to perform this particular analysis. A follow-up study by Holler expanded the original cohort of sibling donor transplants (n=78) with an additional 225 donor/recipient pairs from 4 additional European centers. The association of NOD2 mutations and adverse outcomes was again confirmed and the findings suggested that gut decontamination attenuated the effect of NOD2 mutations on TRM [24]. A further study yet again expanded the study cohort and pooled all the related (n=358) and unrelated (n=342) donor recipient pairs to show that there was a weak association with NOD2/CARD15 variants with GVHD, and there was no overall impact on TRM and OS [12].

The implications of those initial reports, namely that NOD2/CARD15 genotyping might have a role in preemptive stratification of risk profiles in patients undergoing HSCT prompted studies at other centers to validate those findings[14, 26]. van der Velden and colleagues used partially T-depleted grafts in sibling donor transplants and found a significant impact of NOD2/CARD15 polymorphisms on severe acute GVHD and TRM rates when both the donor and recipient had a mutation [26]. A separate study in the UK involving 196 unrelated donor transplants for acute leukemia, the majority of which were T-depleted grafts, also confirmed an increased risk of relapse and overall mortality in recipients with NOD2/CARD15 mutations [14]. While these findings support at least some of the earlier conclusions linking NOD2/CARD15 polymorphisms to worse outcomes following transplantation, other work has reported less consistent findings. Studies have revealed, for example, that donor NOD2/CARD15 mutations led to an unexpected *reduction* in acute GVHD, whereas both donor and recipient NOD2/CARD15 mutations led to the highest incidence of severe GVHD and gut GVHD [13]. Granell and colleagues found an increased risk of relapse and death in patients with NOD2/CARD15 variants undergoing T-cell depleted sibling transplants for acute leukemia with those outcomes being independent of the development of acute or chronic GVHD [22]. Yet another study, involving 198 unrelated and sibling transplants, revealed no association between NOD2/CARD15 variants and negative outcomes following HSCT [15]. Clearly subtle differences in practice patterns in these various centers may account for the variance in the findings, but the findings suggest at the very least there is uncertainty regarding the association between NOD2/CARD15 mutations and the outcomes of HSCT.

In attempting to reconcile our findings with these earlier studies, we can point to some important differences between our study and previous reports. First, the current study group had a lower incidence of NOD2/CARD15 variants, which may have led to our cohort being underpowered to detect a difference in outcome. Among the reasons for the lower mutant frequencies are that our patients were drawn from a more ethnically diverse population, which included 6.7% racial/ethnic minorities. Previous epidemiologic studies have demonstrated that mutations in the NOD2/CARD15 gene are extremely rare outside the Caucasian population [8]. Secondly, our observed rates of GVHD were also lower than earlier studies, which may reflect our exclusion of any donor-recipient mismatched pairs and higher proportion of bone marrow as the stem cell source as opposed to peripheral blood stem cells. Although peripheral blood stem cells are used more commonly today, the inclusion period of this study (1995-2004) dates back to when bone marrow was still the more common source of stem cells and the number of bone marrow transplants in our cohort exceeded peripheral blood stem cell transplants until 2003. Future studies will be required to address the outcomes using predominantly or exclusively peripheral blood stem cells. Lastly, it is likely that NOD2/CARD15 effects may be more pronounced if donor T-cells are strongly suppressed as originally suggested[12] and recently reported[26]

It is also worth noting that the use of T-replete grafts, unrelated donors, and lack of gut decontamination as employed in the current study are factors that one would typically associate with a higher incidence of GVHD. In particular, given the emerging role of luminal flora in the pathogenesis of IBD [27]one would have predicted a priori that the lack

of gut decontamination would if anything exacerbate the incidence of GVHD. However, this was not the case. It is not routine practice in U.S. transplant centers to use gut decontamination as was performed in the majority of the aforementioned studies from European centers, but the exact frequency is unknown and not recorded in the NMDP database.

In designing this study, we selected several IBD markers with a plausible role in GVHD. The findings in regard to IRGM, which has not previously been studied in relationship to HSCT outcomes, have yielded provocative but as yet inconclusive results. Our findings suggest that IRGM variant status did trend towards improved overall survival, disease-free survival, and transplant-related mortality. Nevertheless, due to the multiple variables examined, we selected a p-value of <0.01 to meet statistical significance and we conclude that further study will be required to confirm and extend these associations. Several other IBD-associated genes have recently been elucidated and these may also merit examination in the future to determine if there is a role for polymorphic variants in the development of GVHD. In this context, it is worth emphasizing that the use of non-HLA genes in association studies of transplant outcome should be interpreted with caution, and in the context of biological plausibility, in order to justify further analysis of a particular inflammatory cytokine. By way of example, mouse genetic studies may serve as an invaluable tool in this regard as the findings from NOD2/CARD15 knockout mice point to a key role in innate intestinal immunity[28] and other recent findings which suggest that genetic deletion of the p19 dimer of IL23 may be protective against GVHD[29].

In conclusion, due to the lack of any clear association between the studied variant SNPs and outcomes following HSCT, our data do not support the routine use of NOD2/CARD15, IL-23R, or IRGM polymorphism screening in the patient-donor selection process for HSCT. Further studies are needed to determine if these polymorphisms would have an effect in sibling donor transplants, a population not included in this study, or in exclusively peripheral blood stem cell-based transplants in the U.S.

ACKNOWLEDGEMENTS

This work was supported in part by grants (Digestive Disease Research Core Center DK-52574), HL-38180, DK-56260 (to NOD). The authors are grateful to Dr. Anne Bowcock for helpful discussion and assistance with the Sequenom assays and Dr. Effie Petersdorf for discussions and insights regarding the role of NOD2/CARD15 mutants.

The CIBMTR is supported by Public Health Service Grant/Cooperative Agreement U24-CA76518 from the National Cancer Institute (NCI), the National Heart, Lung and Blood Institute (NHLBI) and the National Institute of Allergy and Infectious Diseases (NIAID); a Grant/Cooperative Agreement 5U01HL069294 from NHLBI and NCI; a contract HHSH234200637015C with Health Resources and Services Administration (HRSA/DHHS); two Grants N00014-06-1-0704 and N00014-08-1-0058 from the Office of Naval Research; and grants from AABB; Aetna; American Society for Blood and Marrow Transplantation; Amgen, Inc.; Anonymous donation to the Medical College of Wisconsin; Astellas Pharma US, Inc.; Baxter International, Inc.; Bayer HealthCare Pharmaceuticals; Be the Match Foundation; Biogen IDEC; BioMarin Pharmaceutical, Inc.; Biovitrum AB; BloodCenter of Wisconsin; Blue Cross and Blue Shield Association; Bone Marrow Foundation; Canadian Blood and Marrow Transplant Group; CaridianBCT; Celgene Corporation; CellGenix, GmbH; Centers for Disease Control and Prevention; Children's Leukemia Research Association; ClinImmune Labs; CTI Clinical Trial and Consulting Services; Cubist Pharmaceuticals; Cylex Inc.; CytoTherm; DOR BioPharma, Inc.; Dynal Biotech, an Invitrogen Company; Eisai, Inc.; Enzon Pharmaceuticals, Inc.; European Group for Blood and Marrow Transplantation; Gamida Cell, Ltd.; GE Healthcare; Genentech, Inc.; Genzyme Corporation; Histogenetics, Inc.; HKS Medical Information Systems; Hospira, Inc.; Infectious Diseases Society of America; Kiadis Pharma; Kirin Brewery Co., Ltd.; The Leukemia & Lymphoma Society; Merck & Company; The Medical College of Wisconsin; MGI Pharma, Inc.; Michigan Community Blood Centers; Millennium Pharmaceuticals, Inc.; Miller Pharmacal Group; Milliman USA, Inc.; Miltenyi Biotec, Inc.; National Marrow Donor Program; Nature Publishing Group; New York Blood Center; Novartis Oncology; Oncology Nursing Society; Osiris Therapeutics, Inc.; Otsuka America Pharmaceutical, Inc.; Pall Life Sciences; PDL BioPharma, Inc; Pfizer Inc; Pharmion Corporation; Saladax Biomedical, Inc.; Schering Corporation; Society for Healthcare Epidemiology of America; StemCyte, Inc.; StemSoft Software, Inc.; Sysmex America, Inc.; Teva Pharmaceutical Industries;; THERAKOS, Inc.; Thermogenesis Corporation; Vidacare Corporation; Vion Pharmaceuticals, Inc.; ViraCor Laboratories; ViroPharma, Inc.; and Wellpoint, Inc. The views expressed in this article do not reflect the official policy or position of the National Institute of Health, the Department of the Navy, the Department of Defense, or any other agency of the U.S. Government.

AUTHOR CONTRIBUTIONS

Y. Nguyen designed the research plan and wrote the paper. A. Al-Lehibi and E. Gorbe performed the sequencing. Ellen Li assisted with design of assays and provided data

interpretation. Michael Haagenson contributed to editing the paper, analyzed data, and performed statistical analysis. Tao Wang performed statistical analysis. S.Spellman and S. Lee provided editorial assistance. N.O. Davidson devised project concept, wrote, and edited the paper.

References

1. Potter V, Moore J. Randomised trials of Graft versus Host Disease prophylaxis in haemopoietic stem cell transplantation. *Rev Recent Clin Trials.* May 2008;3(2):130-138.
2. 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.* May 1 2000;95(9):2754-2759.
3. Macfarlane S, Steed H, Macfarlane GT. Intestinal bacteria and inflammatory bowel disease. *Crit Rev Clin Lab Sci.* 2009;46(1):25-54.
4. Budarf ML, Labbe C, David G, Rioux JD. GWA studies: rewriting the story of IBD. *Trends Genet.* Mar 2009;25(3):137-146.
5. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol.* Jun 2008;8(6):458-466.
6. Parkes M, Barrett JC, Prescott NJ, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet.* Jul 2007;39(7):830-832.
7. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature.* Jun 7 2007;447(7145):661-678.
8. Hugot JP, Zaccaria I, Cavanaugh J, et al. Prevalence of CARD15/NOD2 mutations in Caucasian healthy people. *Am J Gastroenterol.* Jun 2007;102(6):1259-1267.
9. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature.* May 31 2001;411(6837):599-603.
10. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature.* May 31 2001;411(6837):603-606.
11. 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.* Aug 1 2004;104(3):889-894.
12. Holler E, Rogler G, Brenmoehl J, et al. The role of genetic variants of NOD2/CARD15, a receptor of the innate immune system, in GvHD and complications following related and unrelated donor haematopoietic stem cell transplantation. *Int J Immunogenet.* Aug 2008;35(4-5):381-384.
13. 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.* Jan 27 2006;81(2):247-254.
14. 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.* Sep 20 2007;25(27):4262-4269.
15. Sairafi D, Uzunel M, Remberger M, Ringden O, Mattsson J. No impact of NOD2/CARD15 on outcome after SCT. *Bone Marrow Transplant.* Jun 2008;41(11):961-964.

16. Massey DC, Parkes M. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy*. Nov-Dec 2007;3(6):649-651.

17. Dubinsky MC, Wang D, Picornell Y, et al. IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. *Inflamm Bowel Dis.* May 2007;13(5):511-515.

18. Elmaagacli AH, Koldehoff M, Landt O, Beelen DW. Relation of an interleukin-23 receptor gene polymorphism to graft-versus-host disease after hematopoietic-cell transplantation. *Bone Marrow Transplant.* May 2008;41(9):821-826.

19. 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.* Dec 2009;15(12):1571-1577.

20. 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.* Oct 1974;18(4):295-304.

21. Karanes C, Nelson GO, Chitphakdithai P, et al. Twenty years of unrelated donor hematopoietic cell transplantation for adult recipients facilitated by the National Marrow Donor Program. *Biol Blood Marrow Transplant.* Sep 2008;14(9 Suppl):8-15.

22. Granell M, Urbano-Ispizua A, Arostegui JI, et al. Effect of NOD2/CARD15 variants in T-cell depleted allogeneic stem cell transplantation. *Haematologica.* Oct 2006;91(10):1372-1376.

23. Przepiorka D, Anderlini P, Saliba R, et al. Chronic graft-versus-host disease after allogeneic blood stem cell transplantation. *Blood.* Sep 15 2001;98(6):1695-1700.

24. 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.* May 15 2006;107(10):4189-4193.

25. Beelen DW, Elmaagacli A, Muller KD, Hirche H, Schaefer UW. Influence of intestinal bacterial decontamination using metronidazole and ciprofloxacin or ciprofloxacin alone on the development of acute graft-versus-host disease after marrow transplantation in patients with hematologic malignancies: final results and long-term follow-up of an open-label prospective randomized trial. *Blood.* May 15 1999;93(10):3267-3275.

26. 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.* Feb 16 2009.

27. Kucharzik T, Maaser C, Lugering A, et al. Recent understanding of IBD pathogenesis: implications for future therapies. *Inflamm Bowel Dis.* Nov 2006;12(11):1068-1083.

28. Kobayashi KS, Chamaillard M, Ogura Y, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science.* Feb 4 2005;307(5710):731-734.

29. Thompson JS, Chu Y, Glass JF, Brown SA. Absence of IL-23p19 in donor allogeneic cells reduces mortality from acute GVHD. *Bone Marrow Transplant.* Aug 31 2009 [Epub].

Table 1. Characteristics of patients with AML, ALL, CML or MDS in early disease stage receiving either a BM or PBSC first transplant between 1995-2004 with a myeloablative conditioning regimen and having an allele-level matched donor at HLA-A, -B, -C, -DRB1 and -DQB1 from the National Marrow Donor Program

Variable	N Eval	N (%)
Number of patients		390
Number of centers		85
Age, median (range), years	390	37 (<1-59)
Age at transplant	390	
0-9 y		8 (2)
10-19 y		44 (11)
20-29 y		69 (18)
30-39 y		107 (27)
40-49 y		113 (29)
50 and older		49 (13)
Male sex	390	211 (54)
Karnofsky prior to transplant > 90	382	329 (86)
Disease at transplant	390	
AML		64 (16)
ALL		51 (13)
CML		244 (63)
MDS		31 (8)
Graft type	390	
Bone marrow		317 (81)
Peripheral blood		73 (19)
Conditioning regimen	390	
Myeloablative		384 (98)
TBI/Cy		287 (74)
Bu/Cy		75 (19)
TBI		11 (3)
Melphalan		4 (1)
Bu (reduced-intensity)/Cy		4 (1)
Bu		3 (1)
Non-myeloablative		
Cy/Flu		6 (2)
GVHD Prophylaxis	390	
Tacrolimus ± other		124 (32)

Cyclosporine + Methotrexate ± other		256 (66)
Cyclosporine ± other (No Methotrexate)		10 (3)
Donor/Recipient sex match	390	
Male → Male		161 (41)
Male → Female		96 (25)
Female → Male		50 (13)
Female → Female		83 (21)
Donor/Recipient Cytomegalovirus match	390	
Negative/Negative		157 (40)
Negative/Positive		97 (25)
Positive /Negative		53 (14)
Positive / Positive		72 (18)
Unknown		11 (3)
Donor age, median (range), years	390	36 (19-59)
Donor age	390	
< 20		2 (1)
20-29		107 (27)
30-39		146 (37)
40-49		104 (27)
50 and older		31 (8)
Year of transplant	390	
1995		33 (8)
1996		31 (8)
1997		47 (12)
1998		53 (14)
1999		45 (12)
2000		60 (15)
2001		39 (10)
2002		29 (7)
2003		27 (7)
2004		26 (7)
Median follow-up of survivors, mo		77 (12-149)

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; HLA, human leukocyte antigen; TBI, total body irradiation; Bu, busulfan; Flu, fludarabine; Cy, cyclophosphamide

Table 2. Incidence of CARD15, IL-23R, and IRGM SNPs in donors, recipients, or both

Variable	N Eval	N (%)
CARD15	390	
Donor Negative / Recipient Negative		279 (72)
Donor Positive / Recipient Negative		44 (11)
Donor Negative / Recipient Positive		55 (14)
Donor Positive / Recipient Positive		12 (3)
IL-23R	353	
Donor Negative / Recipient Negative		263 (75)
Donor Positive / Recipient Negative		35 (10)
Donor Negative / Recipient Positive		44 (12)
Donor Positive / Recipient Positive		11 (3)
IRGM	385	
Donor Negative / Recipient Negative		229 (60)
Donor Positive / Recipient Negative		66 (17)
Donor Negative / Recipient Positive		66 (17)
Donor Positive / Recipient Positive		24 (6)

Table 3. Overall incidence of evaluated clinical outcomes (N=390)

Clinical outcome	% (95% CI)
100 day (acute)	
Grade III-IV GVHD	21 (17-25)
100 day GI GVHD	
Grade II-IV	14 (11-18)
Grade III-IV	8 (5-11)
1-year chronic GVHD	53 (48-58)
Rate of relapse	
100 days	2 (1-4)
6 months	5 (3-8)
1 year	10 (7-13)
Overall survival	
100 days	85 (82-89)
6 months	77 (73-81)
1 year	67 (62-72)
Disease-free survival	
100 days	84 (80-88)
6 months	74 (70-78))
1 year	63 (58-68)

Table 4. SNP variant status and relative risks of graft versus host disease compared with wild type pairs

IBD-marker SNP	Grade II-IV Acute GVHD	Grade III-IV Acute GVHD	Grade II-IV GI GVHD	Grade III-IV GI GVHD	Chronic GvHD
CARD15 RR CI P value	0.99 [0.68-1.42] 0.93	0.79 [0.43-1.46] 0.45	1.18 [0.61-2.28] 0.63	1.43 [0.61-3.36] 0.41	1.02 [0.70-1.48] 0.93
IL23R RR CI P value	0.74 [0.49-1.13] 0.16	0.66 [0.33-1.34] 0.25	1.10 [0.54-2.26] 0.79	1.10 [0.42-2.91] 0.84	0.84 [0.56-1.25] 0.39
IRGM RR CI P value	0.97 [0.70-1.34] 0.85	0.82 [0.47-1.42] 0.48	1.38 [0.76-2.52] 0.29	1.88 [0.84-4.20] 0.13	1.14 [0.83-1.56] 0.42
Combined SNPs RR CI Pvalue	0.85 [0.65-1.13] 0.26	0.79 [0.51-1.25] 0.32	1.45 [0.85-2.46] 0.17	2.05 [0.97-4.33] 0.062	1.0 [0.76-1.32] 1.0

Table 5. Risk of + vs. – variant status and relative risks of clinical outcomes compared with wild type pairs

IBD-marker SNP	Overall Survival	Disease- Free Survival	Transplant-related mortality	Relapse
CARD15				
RR	0.83	0.81	0.86	0.73
CI	[0.54-1.28]	[0.54-1.24]	[0.53-1.39]	[0.30-1.74]
P value	0.41	0.34	0.54	0.47
IL23R				
RR	1.35	1.38	1.24	1.60
CI	[0.91-2.02]	[0.93- 2.07]	[0.78-1.98]	[0.75-3.41]
P value	0.14	0.11	0.36	0.23
IRGM				
RR	0.64	0.68	0.59	0.99
CI	[0.43-0.95]	[0.46-0.99]	[0.37-0.95]	[0.51-1.92]
P value	0.03	0.04	0.03	0.97
Combined SNPs				
RR	0.77	0.80	0.74	0.97
CI	[0.57-1.05]	[0.59-1.08]	[0.53-1.06]	[0.54-1.74]
P value	0.09	0.14	0.09	0.92

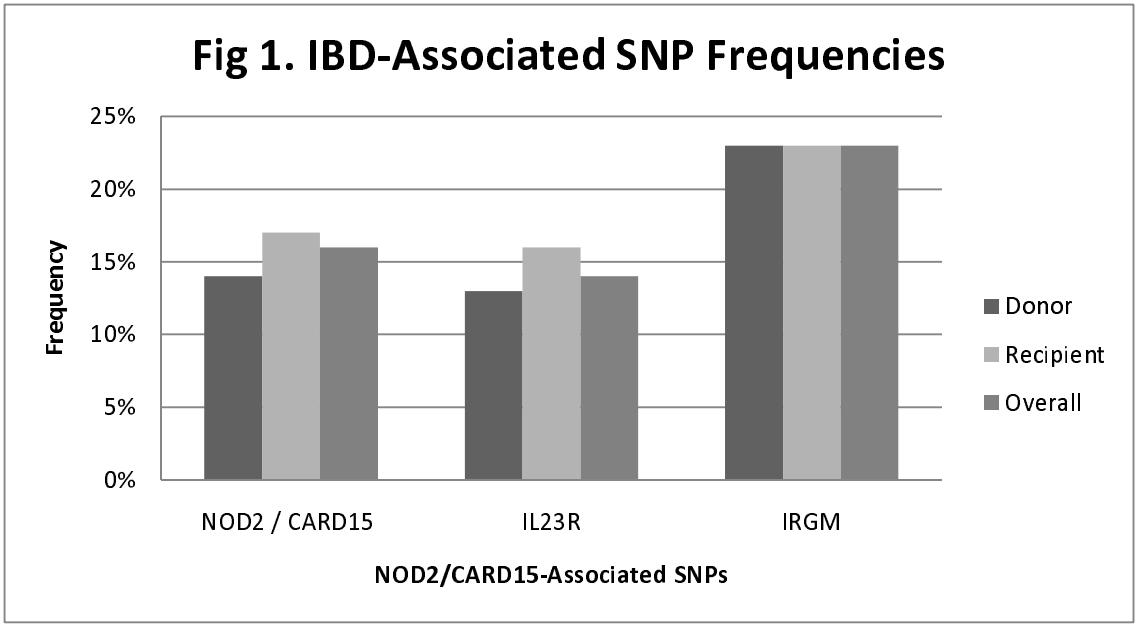
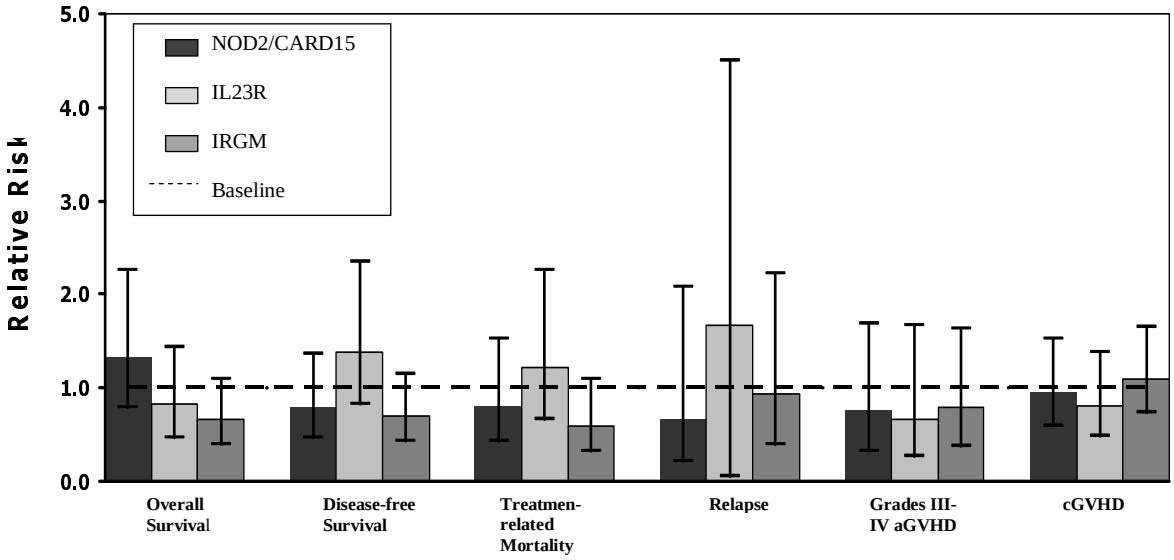


Fig 2. Adjusted Relative Risks of Patient IBD-SNP + vs. – Variant Status



Prepublished online February 22, 2010;
doi:10.1182/blood-2009-09-243840

Insufficient Evidence for Association of NOD2/CARD15 or Other Inflammatory Bowel Disease-Associated Markers on GVHD Incidence or Other Adverse Outcomes in T-Replete, Unrelated Donor Transplantation

Yume P. Nguyen, Abed Al-Lehibi, Elizabeth Gorbe, Ellen Li, Michael Haagenson, Tao Wang, Stephen Spellman, Stephanie J. Lee and Nicholas O. Davidson

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.