TRANSPLANTATION

Recipient Tumor Necrosis Factor-α and Interleukin-10 Gene Polymorphisms Associate With Early Mortality and Acute Graft-Versus-Host Disease Severity in HLA-Matched Sibling Bone Marrow Transplants

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The proinflammatory cytokine tumor necrosis factor-α (TNF-α) is strongly implicated in graft-versus-host disease (GVHD) and other acute bone marrow transplant (BMT) complications. The antiinflammatory interleukin-10 (IL-10) antagonizes TNF-α and reduces GVHD. We previously showed association of recipient TNF (TNFd) and IL-10 (IL-10^{-1064}) gene polymorphisms with acute GVHD severity in matched sibling BMT using only cyclosporin A monotherapy. The current study tested association of GVHD with TNFd and IL-10$^{-1064/-1082}$ polymorphisms in a large cohort (144 matched sibling donor/recipient pairs) given both cyclosporine A (CyA) and methotrexate (MTX) prophylaxis. Genotype results were correlated with acute and chronic GVHD and mortality. Patients homozygous for the TNFd microsatellite allele 3 had higher early mortality: 23.7% of TNFd3/d3 homozygotes died before day 30, compared with 6.80% of non-d3/d3 recipients ($P = .013$). Recipients possessing longer IL-10^{-1064} microsatellite alleles developed more severe acute GVHD: 22.3% of recipients possessing alleles 12 to 15 developed grade III to IV GVHD, versus 3.92% of those with smaller alleles ($P < .01$). Other recipient or donor genotypes tested did not significantly affect GVHD or mortality. We conclude that recipient TNFd and IL-10^{-1064} polymorphisms associate with early mortality and severe acute GVHD in matched sibling BMT with dual prophyaxis. This supports the hypothesis of genetic predisposition towards GVHD and other BMT complications other than histocompatibility antigen disparity.

GRAFT-VERSUS-HOST DISEASE (GVHD) remains the most common allogeneic bone marrow transplant (BMT) complication; mild acute GVHD (aGVHD) does not necessarily affect survival, but severe aGVHD, especially grades III to IV, increases mortality,[1] and once established, often responds poorly to therapy.[2] Prophylaxis with immunosuppressive drugs and/or T-cell depletion has side effects of increasing infection and decreasing the graft-versus-leukemia (GVL) effect.[3] Currently there are no widely established approaches to individualized targeting of GVHD prophylaxis.

Cytokines are important GVHD mediators and regulators.[4,5] Although conditioning exerts an antineoplastic effect, increased total body irradiation (TBI) intensity causes accelerated aGVHD and more infections.[6] TBI and chemotherapy damage host cells, releasing proinflammatory cytokines including tumor necrosis factor-α (TNF-α).[7] Gastrointestinal mucosal injury allows bacterial lipopolysaccharide to stimulate monocyte TNF-α secretion.[8] TNF-α increases major histocompatibility complex (MHC) expression,[9] facilitates cell-mediated cytotoxicity and is itself cytotoxic via apoptosis.[10] TNF-α release during conditioning precedes severe aGVHD[1] and other early inflammatory transplant-related complications (TRC), including venoocclusive disease (VOD) and septic shock, and is predictive of high mortality.[11] However, TNF-α release before conditioning is not linked to increased aGVHD, possibly via desensitization.[12] Clinical studies show anti–TNF-α antibodies during conditioning decrease aGVHD,[13] but established aGVHD responds only transiently.[14]

The TNF-α gene maps within the MHC class III region on chromosome 6 near many polymorphisms, several of which associate with inflammatory disease.[15] TNFd, a (GA)n-repeat microsatellite sequence,[16] maps downstream of the TNF-α gene within intron 3 of the nearby leukocyte-specific transcript (LST)-1 gene.[17] The most commonest allele, TNFd3, associates with higher in vitro TNF-α production,[18] and TNFd3/d3 homozygous cardiac-transplant recipients have increased rejection.[19] We recently demonstrated that TNFd3/d3 homozygosity associated with grade III to IV aGVHD in HLA-matched sibling BMT using prophylactic cyclosporine A (CyA) monotherapy.[20]

The antiinflammatory cytokine interleukin-10 (IL-10)[21] inhibits monocyte production of proinflammatory cytokines,[22] including TNF-α, and decreases apoptosis induced by lipopolysaccharide and irradiation.[8] IL-10 also reduces MHC expression and attenuates recognition by cytotoxic lymphocytes.[23] A recently described IL-10–dependent $CD4^+$ T-cell subset, the T-regulatory 1 (Tr1)-cell, can induce antigen-specific tolerance.[24,25] In BMT, IL-10 production preconditioning protects from TNF-α release, aGVHD, and other TRC.[26] Cells from patients with acute or chronic GVHD (cGVHD) produce less IL-10 in vitro.[27,28] The IL-10 gene regulatory region includes 2 microsatellite polymorphisms, which associate with differential in vitro IL-10 production.[29] An excess of allele 13 of the IL-10^{-1064} (CA)n-repeat polymorphism occurs in systemic lupus erythematosus.[30] Recently, we demonstrated that greater IL-10^{-1064} repeat number in the recipient associated with severe aGVHD in CyA-treated matched sibling BMT and that this association operates in parallel to that of TNFd3/d3.[20] The nearby IL-10^{-1082} (G/A)-polymorphism A allele associates with lower in vitro IL-10

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Table 1. BMT Patient Characteristics

Median age	36.08 yr	Range 2-58.9 yr
	SD = 14.13 yr	
Gender	74 M	70F
Diagnosis	CML	50
	AML	50
	MDS	18
	NHL	7
	ALL	6
	CLL	5
	AA	3
	MM	3
	Other	2

Abbreviations: CML, chronic myeloid leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; AA, aplastic anemia; MM, multiple myeloma.

production.[31] Possession of both TNFd3/d3 genotype and IL-10^{-1082} A allele associates with greater cardiac rejection.[19]

The current study was designed to test association of acute and chronic GVHD with TNFd and IL-10^{-1064} genotypes of both donors and recipients in a larger population given combined CyA/methotrexate (MTX) prophylaxis[32] and to examine potential association of the neighboring IL-10^{-1082} polymorphism. Genotypes for TNFd and IL-$10^{-1064/-1082}$ polymorphisms were determined for CyA/MTX-treated matched sibling BMT donor/recipient pairs. These genotypes were correlated with clinical outcomes including GVHD severity and mortality. We hypothesize that recipient genetic factors are important in determining the severity of transplant complications. In this study, we strengthen that hypothesis, showing that recipient TNF and IL-10 polymorphisms associate with early mortality and aGVHD after matched sibling donor BMT.

MATERIALS AND METHODS

BMT patient characteristics. A total of 144 sibling donor/recipient pairs who had undergone HLA-matched (serologically for HLA-A/-B antigens; by high resolution molecular typing for HLA-DRB1) BMT at the University of Minnesota were genotyped, while blinded to clinical outcomes (Table 1). All patients or their guardians signed consent forms approved by the University of Minnesota Institutional Review Board. Conditioning comprised of cyclophosphamide (60 mg/kg × 2) followed by fractionated TBI (165 cGy twice daily for 4 days; total 1,320 cGy); except for 2 patients who received 125 cGy 3 times daily for 3 days and twice daily on the fourth day (total 1,375 cGy), and 4 pediatric patients who received 200 cGy twice daily for 3 days (total 1,200 cGy), followed by cyclophosphamide (60 mg/kg × 2). All grafts were non–T-cell depleted and GVHD prophylaxis consisted of CyA from day −3 (maintaining levels between 200 to 400 ng/mL assayed by high-performance liquid chromatography) and short course MTX (15 mg/m^2 day 1 and 10 mg/m^2 days 3, 6, and 11).

TNF-α and IL-10 genotypes. Donor/recipient genotypes for the TNFd and IL-$10^{-1064/-1082}$ polymorphisms were determined as previously described[16,30,31] using stored DNA or crude cell-lysates where purified DNA was unavailable. Some samples contained insufficient intact DNA to act as polymerase chain reaction (PCR) template for all polymorphisms, despite phenol/chloroform extraction and ethanol precipitation. PCR products were resolved by polyacrylamide gel electrophoresis (8%; 19:1 acrylamide:bisacrylamide) and visualized by silver staining. Control DNA heterozygous for each common allele was used to ensure accuracy. As TNFd is within the MHC complex, TNFd genotype acted as a control for donor/recipient pair matching.

IL-10 haplotypes were determined in an IL-10^{-1082} allele-specific PCR including the IL-10^{-1064} microsatellite in the product, thereby ascertaining the IL-10^{-1064} allele together with its associated IL-10^{-1082} G or A allele. Primers were 5′-AGCAACACTCCTCGTCGCAAC-3′ (JW-F) with 5′-CCTATCCCTACTTCCCCC-3′ (B1) or 5′-CCTATCCCTACTTCCCCT-3′ (B2). Reactions contained 20 μmol/L of each primer, 0.5 U Taq polymerase (Bioline, London, UK) and 200 μmol/L deoxyribonucleoside triphosphates (dNTP) mixture with 1.5 mmol/L $MgCl_2$ in 1x NH_4 Buffer (Bioline) in addition to test DNA, to a final volume of 25 μL. Amplification was performed on Perkin-Elmer thermal-cycler (Norwalk, CT) with 30 cycles of: 94°C for 30 seconds; 60°C for 60 seconds; 72°C for 60 seconds; followed by a final extension of 72°C for 7 minutes.

Statistical analysis. Data were analyzed in contingency tables by Fisher's exact test (other than Kaplan-Meier survival-curve comparison by χ^2), using GraphPad Prism 2 software (GraphPad Software Inc, San Diego, CA), with P values (all 2-sided) less than .05 regarded as statistically significant.

RESULTS

TNFd polymorphism frequencies. A total of 562 TNFd alleles was analyzed; 8 samples could not be typed. Allele frequency distribution did not differ significantly from previous reports,[16,19,20] with homozygote frequencies comparable to those predicted by the Hardy-Weinberg equilibrium (eg, TNFd3/d3: predicted = 72 pairs; observed = 76). One pair exhibited a single TNFd mismatch (donor genotype d3d4; recipient d4d5).

IL-10 polymorphism haplotypes. A total of 574 IL-10^{-1064} alleles was ascertainable; allele frequency distribution did not differ significantly from previous reports,[20,29,30] with comparable homozygote frequencies to those predicted (eg, IL-10^{-1064} i9/i9: predicted = 45 pairs, observed = 51). The IL-10^{-1082} (G/A)-polymorphism was ascertained for 430 alleles, with comparable frequencies to previous reports (Table 2).[19,31] Haplotype analysis showed IL-10^{-1064} alleles possessing greater numbers of dinucleotide repeats, referred to as i(12-16), to be preferentially associated with IL-10^{-1082} A. One hundred sixty-three of 276 IL-10^{-1064} i(7-11) alleles were associated with a G allele at IL-10^{-1082}, and 101 of 154 IL-10^{-1064} i(12-16) alleles associated with IL-10^{-1082} A ($P < .001$). This is consistent with findings from both normal subjects and the previously analyzed Northern UK BMT cohort (P.G.M., unpublished observation, February 1999).

Clinical outcomes. Of the 144 patients, 16 died before day 30, hence 128 were evaluable for aGVHD grade. Twelve of these 16 had sepsis (6 fungal), 6 diffuse alveolar/pulmonary hemorrhage, 3 multisystem organ failure (MSOF), 2 hyperammonemia, 1 acute respiratory distress syndrome, and 1 VOD. Eighty-eight patients developed aGVHD (grading according to Glucksberg et al[33]): 26 grade I, 43 grade II, 17 grade III, and 2 grade IV aGVHD. A further 18 patients died before day 100, leaving 110 evaluable for cGVHD grade. Presence of aGVHD showed a trend toward correlation with cGVHD risk: 11 of 32 patients without aGVHD surviving >100 days developed de

Table 2. IL-$10^{-1064/-1082}$ Haplotypes

	IL-10^{-1064} i(7-11)	IL-10^{-1064} i(12-16)	
IL-10^{-1082} G	163	53	$P < .001$
IL-10^{-1082} A	113	101	

novo cGVHD, while 44 of 78 with grade I to IV aGVHD went on to develop cGVHD ($P = .058$). Of the 55 patients developing cGVHD, 4 had limited and 51 had extensive disease (grading according to Atkinson et al[34]).

Median follow-up duration was 869 days. Overall survival was 47.2% (76 deaths) and showed significant correlation with presence of aGVHD: 62 of 109 (57.8%) patients with grade 0 to II aGVHD survived, compared with 6 of 19 (31.6%) with grade III to IV aGVHD ($P = .049$). cGVHD presence did not correlate with overall survival ($P = 1.0$). If the 6 patients who received nonstandard conditioning were excluded, the rates of mortality and GVHD were no different, as none of these patients died before day 30 nor did they develop severe aGVHD.

Early mortality. TNFd3/d3 genotype (shared by donor and recipient) was significantly associated with early mortality: 23.7% (9 of 37) of TNFd3/d3 homozygotes died before day 30, compared with 6.80% (7 of 96) of nond3/d3 recipients ($P = .013$) (Table 3). Possession of only 1 TNFd3 allele was not associated with early death ($P = .56$). Early death was not associated with recipient status at either IL-10^{-1064} or $^{-1082}$ polymorphism.

Acute GVHD. TNFd3/d3 genotype was not associated with severe aGVHD (grade III to IV) in those surviving more than 30 days ($P = .24$) (Table 4). Recipient IL-10^{-1064} alleles with larger numbers of dinucleotide repeats were significantly associated with severe aGVHD: 22.3% (17 of 76) of recipients possessing 1 or more i(12-16) allele developed grade III to IV aGVHD, while 3.92% (2 of 51) of recipients with only i(7-11) alleles developed grade III to IV aGVHD ($P = .0045$). Recipient IL-10^{-1082} or donor IL-10 genotypes did not associate with aGVHD.

Table 3. Early Death Correlation With Genotype

Genotype	Alive >30 Days	Dead by Day 30	*P* Value
TNFd3/d3*	29	9	$P = .0130$
Non-d3/d3†	96	7	
TNFd3+‡	91	13	$P = .561$
TNFd3−§	34	3	
Donor IL-10^{-1064}			
i(7-11) only	53	6	$P = 1.0$
i(12-16) present	74	10	
Donor IL-10^{-1082}			
GG	17	1	$P = 1.0$
A present	53	6	
AA	24	2	$P = 1.0$
G present	46	5	
Recipient IL-10^{-1064}			
i(7-11) only	51	7	$P = .783$
i(12-16) present	76	9	
Recipient IL-10^{-1082}			
GG	36	5	$P = .529$
A present	88	8	
AA	30	2	$P = .732$
G present	94	11	

*TNFd3/d3 homozygotes.
†TNF genotypes other than d3/d3.
‡Genotypes with 1 or more TNFd3 allele.
§Genotypes without a TNFd3 allele.

Table 4. Acute GVHD Correlation With Genotype

Genotype	aGVHD Grade 0-II	aGVHD Grade III-IV	*P* Value
TNFd3/d3*	27	2	$P = .240$
Non-d3/d3†	80	16	
TNFd3+‡	81	11	$P = .247$
TNFd3−§	26	7	
Donor IL-10^{-1064}			
i(7-11) only	46	7	$P = .800$
i(12-16) present	62	12	
Donor IL-10^{-1082}			
GG	16	1	$P = .670$
A present	46	7	
AA	21	3	$P = .160$
G present	41	16	
Recipient IL-10^{-1064}			
i(7-11) only	49	2	$P = .0045$
i(12-16) present	59	17	
Recipient IL-10^{-1082}			
GG	28	8	$P = .180$
A present	77	11	
AA	27	3	$P = .561$
G present	78	16	

*TNFd3/d3 homozygotes.
†TNF genotypes other than d3/d3.
‡Genotypes with 1 or more TNFd3 allele.
§Genotypes without a TNFd3 allele.

Chronic GVHD. Neither donor nor recipient TNFd or IL-10 polymorphisms were associated with cGVHD (Table 5).

Overall mortality. TNFd3/d3 homozygotes had lower overall survival, but not to a significant degree, despite increased early mortality: 42.1% (16 of 38) of TNFd3/d3 homozygotes were survivors, and 49.5% (51 of 103) non-TNFd3/d3 recipients survived ($P = .45$) (Table 6). Median TNFd3/d3 homozygous recipient survival was 373 days compared with 467 days for non-d3/d3 recipients (Kaplan Meier χ^2, $P = .29$). Recipient IL-10^{-1064} polymorphism length did not correlate with overall survival, despite association with aGVHD severity: 40 recipi-

Table 5. Chronic GVHD Correlation With Genotype

Genotype	cGVHD Absent	cGVHD Present	*P* Value
TNFd3/d3*	14	12	$P = .822$
Non-d3/d3†	40	42	
Donor IL-10^{-1064}			
i(7-11) only	24	18	$P = .327$
i(12-16) present	31	36	
Donor IL-10^{-1082}			
GG	9	5	$P = .363$
A present	21	23	
AA	11	8	$P = .583$
G present	19	20	
Recipient IL-10^{-1064}			
i(7-11) only	19	21	$P = .690$
i(12-16) present	36	33	
Recipient IL-10^{-1082}			
GG	19	13	$P = .300$
A present	36	39	
AA	12	15	$P = .507$
G present	43	37	

*TNFd3/d3 homozygotes.
†TNF genotypes other than d3/d3.

Table 6. Overall Survival Correlation With Genotype

Genotype	Alive	Dead	*P* Value
TNFd3/d3*	16	22	*P* = .434
Non-d3/d3†	51	52	
TNFd3+‡	47	57	*P* = .444
TNFd3−§	20	17	
Donor IL-10^{-1064}			
i(7-11) only	25	34	*P* = .398
i(12-16) present	42	42	
Donor IL-10^{-1082}			
GG	11	7	*P* = .291
A present	27	32	
AA	13	13	*P* = 1.0
G present	25	26	
Recipient IL-10^{-1064}			
i(7-11) only	27	31	*P* = 1.0
i(12-16) present	40	45	
Recipient IL-10^{-1082}			
GG	22	19	*P* = .457
A present	44	52	
AA	18	14	*P* = .31
G present	48	57	

*TNFd3/d3 homozygotes.
†TNF genotypes other than d3/d3.
‡Genotypes with 1 or more TNFd3 allele.
§Genotypes without a TNFd3 allele.

ents with i(12-16) and 27 recipients with only i(7-11) survived, while 45 recipients with i(12-16) and 31 recipients with only i(7-11) died ($P = 1.0$). Recipient IL-10^{-1082} or donor IL-10 polymorphisms did not associate with overall mortality.

DISCUSSION

Preliminary studies led us to hypothesize that recipient cytokine gene polymorphism was related to inflammatory complications of BMT, including GVHD; the results of this study are consistent with this hypothesis. Transplant recipients who were homozygous for the TNFd3 allele had significantly higher early mortality, suggesting genetic susceptibility to fatal acute inflammatory TRC. Of the 9 TNFd3d3 homozygotes dying within the first month, 3 had diffuse alveolar hemorrhage associated with sepsis, 2 developed MSOF in association with severe sepsis, 2 succumbed to fungal infection, 1 to respiratory syncitial virus pneumonia, and 1 to Citrobacter septicemia. TNF-α release is implicated in pathogenesis of aGVHD, VOD, MSOF, and septic shock[35] The association of fatal early TRC and TNFd3d3 polymorphism is consistent with the hypothesis that recipient genetic variation in cytokines influences BMT outcome. The association between TNFd3/d3 genotype and inflammatory TRC including aGVHD probably reflects increased TNF-α release as demonstrated in cardiac allograft recipients.[18] However, the TNFd microsatellite lies within the neighboring LST-1 gene, the function of which is unknown, and another mechanism cannot currently be excluded. As the study was retrospective, no TNF-α levels were available.

In our previous study using CyA monotherapy, TNFd3/d3 homozygotes had increased severe aGVHD.[20] In this current cohort given combined CyA/MTX prophylaxis, TNFd3/d3 homozygotes who survived the initial month did not exhibit more severe aGVHD. The differences observed between the 2 studies may reflect increased prophylaxis (overall rate of grade III to IV aGVHD 22.4% in the monotherapy study compared with 14.8% in the current cohort given CyA/MTX). The addition of MTX may have reduced TNF-α's influence on aGVHD without modulating that of IL-10, which is not mediated solely via TNF-α-antagonism; similar differential effects of MTX on cytokine release have been described in systemic lupus erythematosus.[36] Alternatively, TNFd3/d3 homozygotes who died early might otherwise have subsequently developed severe aGVHD. The previous study was not designed to assess early transplant outcomes, as only those recipients who had aGVHD grade ascertainable were analyzed.[20]

TNFd3d3 homozygous recipients' overall median survival was decreased, but not to a significant degree, despite a significantly increased early mortality. This may reflect the fact that other causes of death such as relapse make a large contribution to overall survival, and the fact that TNFd3d3 homozygotes constitute only 25% of the population. Knowledge of a genetic predisposition to inflammatory TRC might aid pretransplant assessment and counselling. TNFd genotyping may facilitate targeting of experimental TNF-α antagonists, such as anti–TNF-α antibodies[13] or soluble-TNF-receptors,[7] toward recipients with the high-risk TNFd3/d3 genotype. Alternatively, recipients might benefit from alteration in conditioning; however, as only 6 patients differed from the routinely used TBI dose, it is not possible to determine if the association of TNFd3d3 with TRC was dependent on the conditioning regimen.

Recipients possessing IL-10^{-1064}alleles with greater (CA)n repeat numbers had more severe aGVHD, in agreement with previous findings in recipients given CyA alone.[20] The associations of TNFd and IL-10^{-1064} genotype with grade III to IV aGVHD demonstrated in the CyA monotherapy BMT cohort could be combined to show cumulative risk of severe GVHD.[20] However, in the current study, TNFd3 homozygosity was associated with fatal early TRC and hence assessment of TNFd3d3 in conjunction with IL-10^{-1064} genotype in relation to aGVHD was not possible. No association of either of the IL-10 polymorphisms tested with GVHD was found in donors.

Linkage of the IL-10 polymorphisms examined to neighboring genes is unlikely to account for the observed relationship, as no other genes currently implicated in GVHD map to chromosome 1q. In normal subjects, the IL-10^{-1082} polymorphism A allele associates with lower in vitro IL-10 production by concanavalin A–stimulated lymphocytes.[31] IL-10 haplotyping (Table 2) indicates that longer IL-10^{-1064} alleles associate preferentially with the IL-10^{-1082} A allele. Similar alleleic linkage has been observed in other populations (P.G.M., unpublished observation, February 1999). Hence, longer IL-10^{-1064} alleles may associate with lower in vitro lymphocyte IL-10 release. However, the IL-10^{-1064} allele 14 is reported to associate with higher in vitro IL-10 release from lipopolysaccharide-stimulated whole blood.[29] Differences in composition of cell population and stimulating mitogens make extrapolation from such in vitro to in vivo data difficult.

cGVHD did not associate with either IL-10 polymorphism, despite cGVHD usually occuring after preceeding aGVHD.[37] Although cGVHD has been linked to reduced IL-10,[27]

IL-$10^{-1064/-1082}$ genotype is not informative with respect to cGVHD in this study.

Pretransplant IL-10^{-1064} genotyping may allow more individually tailored prophylaxis, with increased immunosuppression administered only to patients with high aGVHD risk-associated genotypes. Typing recipients may aid decisions regarding those who could benefit most from experimental antiinflammatory cytokines such as recombinant IL-10.[38] Reduced prophylaxis for recipients with low aGVHD risk-associated genotypes transplanted for malignancy might permit enhanced GvL effect, hence reducing risk of relapse without adding to aGVHD morbidity and mortality.

A combination of established GVHD risk factors in HLA-matched siblings, such as skin explant analysis,[39] minor histocompatibility antigen incompatibility,[40] and herpes/cytomegalovirus status,[1] with genotyping for TNF and IL-10 polymorphisms (and other potential genotypic risk factors) could allow the creation of a GVHD risk index. Such an index would facilitate accurate individual risk calculation, permitting adjustment of prophylaxis accordingly. Although IL-10 genotype's influence on aGVHD has been demonstrated in cohorts given differing prophylaxis, further studies of both TNF and IL-10 polymorphisms' role in modulating GVHD will be important in building such a risk index, together with investigation of other candidate immunogenetic polymorphisms.

Our findings support the hypothesis that recipient response during BMT conditioning is critical in subsequent outcome and that this involves a substantial genetic component. Recipient cytokine genotype could be used to guide more appropriate GVHD prophylaxis, both established and experimental, particularly in combination with other risk factors.

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