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LETTER TO THE EDITOR

Mannan-binding lectin deficiency attenuates acute GvHD in pediatric hematopoietic stem cell transplantation

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Mannan-binding lectin (MBL) is an acute phase protein that is synthesized in the liver and involved in first-line immune defense. Together with MBL-associated serine proteases (MASPs), MBL mediates opsonization and activates the lectin pathway of C′. The MBL gene is highly polymorphic and specific single-nucleotide polymorhism (SNP) patterns define at least 7 common haplotypes, giving 28 genotype possibilities. Exon-1 and/or promoter SNPs affect MBL serum levels and/or function (reviewed in ref. 1). Particular SNPs and/or low serum levels are listed among common human immunodeficiencies but only rarely translate into a severe clinical phenotype.[1] Nonetheless, in hematopoietic stem cell transplantation (HSCT), a setting that *per se* causes a transient

Table 1. Correlation of MBL and HSCT outcome

		MBL serum levels		
		< 300 ng/mL	*> 300 ng/mL*	P-*value*
Cases		12	76	
outcome	2-yrs pSU	0.92 (0.54–0.99)	0.70 (0.57–0.80)	0.28
MAC	*N*	5	55	
	2-yrs pSU	0.80 (0.20–0.97)	0.69 (0.53–0.81)	0.86
RIC	*N*	7	25	
	2-yrs pSU	1 (0 events)	0.75 (0.50–0.88)	0.19
Infections	Bacterial	8	56	0.73
	Viral	11	70	1
	Fungal	1	25	0.1
Sepsis	Yes/susp	7	37	
	No	5	39	0.47
ICU admission	Yes	1	16	
	No	11	60	0.31
Cause of death	TRM	1	12	0.56
	DOD	0	7	0.30

		MBL genotype groups				
		Very low	*Low*	*Medium*	*High*	P-*value*
Cases		3	17	20	56	
Outcome	2-yrs pSU	1	0.72 (0.41–0.89)	0.48 (0.25–0.68)	0.69 (0.53–0.80)	0.10
Infections	Bacterial	1	13	18	39	0.74
	Viral	3	16	19	50	0.15
	Fungal	1	2	9	18	0.5
Sepsis	Yes/susp	2	7	11	33	
	No	1	10	9	23	0.25
ICU admission	Yes	0	2	7	15	
	No	3	15	13	41	0.61

Abbreviations: 2-yrs pSU = *P*-value of 2-year survival; DOD = dead of disease; HSCT = hematopoietic stem cell transplantation; ICU = intensive care unit; MAC = myeloablative conditioning; MBL = mannan-binding lectin; RIC = reduced intensity conditioning; susp = suspected; TRM = transplant-related mortality. For classification of MBL genotypes, see Supplementary Information. The *P*-value for survival analysis, MAC and RIC indicates the significance value comparing entire survival curves calculated by the use of log-rank test. The *P*-value for MBL genotype groups indicates the significance value comparing MBL high/medium versus low/very low groups.

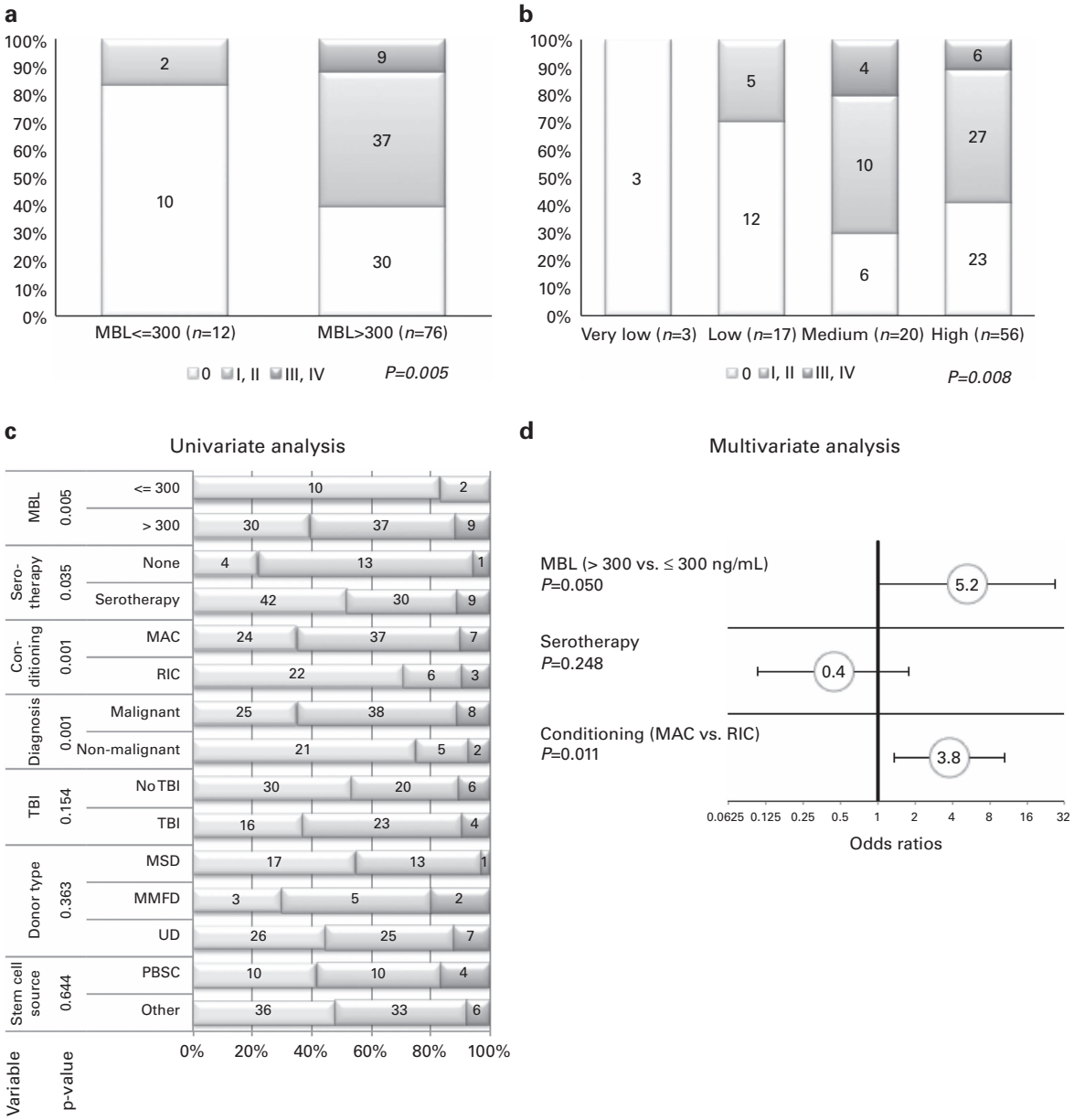

Figure 1. The attenuating impact of MBL deficiency on acute GvHD. The shading of the bars (□ ▨ ▩) indicate the grade of GvHD. The height of the bars indicates the percentage of the respective grade and the inserted numbers indicate the number of patients with GvHD. (**a**) Comparison of the GvHD risk by MBL serum levels and (**b**) by combined low/very low and high/medium genotypes. (**c**) Univariate analysis of variables potentially impacting on GVHD. (**d**) Multivariate analysis including variables significantly affecting GVHD in univariate analyses. Note that because RIC and non-malignant diseases were highly correlated, only the conditioning has been included in the model. A model including malignant versus non-malignant disease gives similar results. Error bars indicate the 95% confidence interval.

but profound state of immunodeficiency, low MBL levels or particular MBL genotypes are reported to contribute to and increase the rate of transplant-related infections.

In consideration of the so far inconsistent findings of the role of MBL in HSCT in adult,[2–5] as well as pediatric[6] HSCT, we prospectively investigated the impact of MBL levels, genotypes and MASP-2 levels in a series of 99 pediatric ($n = 96$) and young adult ($n = 3$) HSCT recipients. MBL deficiency was defined by MBL levels < 300 ng/mL and/or particular MBL haplotypes. Patient characteristics, MBL genotype data and corresponding MBL levels are summarized in the Supplementary Information together with MASP-2 serum levels and statistical analyses.

RESULTS

The *P* of a 2-year overall survival (2-yrs pSU) of the entire cohort of 99 transplant recipients was 67%. We found that neither low MBL serum levels nor MBL genotypes that are usually linked with a low serum level or decreased protein function[1] were associated with a poor outcome (Table 1).

We also did not note any increased risks for bacterial, viral or fungal infections in MBL deficient patients that would have required their admission to intensive care units within a 100 day post-transplantation period (Table 1). Setting the threshold to < 500 ng mL^{-1} equally failed to show any negative impact. These observations are in line with those obtained in a similar previous study of pediatric HSCT patients, in which MBL levels of

< 400 ng/mL were also not associated with an increased infection rate.[6] The findings in studies investigating adult-HSCT recipients, on the other hand, are inconsistent. In those, low MBL levels had either no effect on the risk of infection[3] or, they had even a negative impact on the infection risk under particular circumstances, for instance in case of TBI.[2,4] The uniform finding was that in neither of the studies, like in ours, MBL low serum levels and/or MBL particular genotypes affected the ultimate outcome.

However, the novel and most intriguing finding in the present study is the significantly reduced rate of acute GvHD (aGvHD) in MBL deficient patients, irrespective of whether MBL deficiency was defined by serum levels or genotypes (Figures 1a and b). Of the 12 patients with MBL levels ⩽300 ng/mL, only 2 developed mild (grade I–II) aGvHD. In contrast, 46/76 (61%) patients with MBL levels of >300 ng/mL developed aGvHD, 37 (48%) of them grade I–II and 9 (12%) grade III–IV aGvHD ($P=0.005$). Moreover, the *P*-value of aGvHD was also significantly lower ($P=0.008$) in the combined genotype-defined low and very low level MBL groups than in the medium/high genotype ones. Univariate analyses of relevant transplant-related GVHD risk variables revealed that the use of serotherapy and reduced intensity conditioning (RIC), which was almost exclusively used in patients with non-malignant diseases, were associated with a decreased risk of GvHD (Figure 1c and Supplementary Information). Apart from RIC regimen, MBL levels of ⩽300 ng/mL was the only parameter that remained statistically valid in a multivariate model of logistic regression analysis (Figure 1d).

The finding of a statistically significant inverse correlation of low MBL levels and the incidence and severity of aGvHD may suggest a substantial role of MBL-mediated C′ activation in the pathophysiology of GvHD. There is indeed accumulating evidence for a prominent function of C′ activation in allo-reactivity.

A reduced C′ activation was proposed previously to be an important factor in the ameliorated risk of transplant rejection in MBL deficient patients receiving solid organs, such as kidney, heart and lung.[7] Allo-reactivity is understood to be an inflammatory process with ensuing C′ activation that enhances the extent of tissue damage. This view of MBL's role in inflammatory processes is also corroborated by the better outcome of MBL deficient patients with ischemic events, such as myocardial infarction or ischemic stroke, in which reduced collateral tissue injury on the basis of reduced C′ activation was suggested to co-determine the better course and final outcome.[7,8] Along this line of argument, a substantial role of C′ activation in GvHD has also been supported experimentally in a murine HSCT model. C′ deficient mice were found to have a significantly reduced GvHD-associated morbidity and mortality,[9] most likely because of a diminished Th1/Th17 polarization upon C′ inhibition, recently also shown in human T-cells.[10]

In human HSCT, both the conditioning regimen and the invasion of donor allo-reactive T-cells into target tissues have an effect on the extent of tissue injury and C′ activation. The binding of MBL to apoptotic and necrotic cells[11] in the process of clearing damaged cells may enhance C′ activation and thus contribute to GvHD severity. Conversely, both low MBL levels, as well as specific MBL genotypes, in which the altered secondary structure of the collagenous region of MBL weakens MBL binding to MASPs, result in an impaired activation of the lectin pathway (reviewed in ref. 1) and might reduce the development and progression of GvHD.

Taken together, MBL deficiency appears to be beneficial in diseases in which the pathology is driven by C′ activation that aggravates tissue damage. Although the limited number of patients together with the heterogenous transplant procedures do not allow us to draw a final conclusion about the specific contribution of MBL in this setting, low MBL levels do not seem to increase the risk of severe infections in the immediate post-transplantation period of pediatric and young adult-HSCT recipients, but rather diminish the rate and severity of aGvHD.

Although the biological and clinical effects of MBL deficiency in HSCT clearly deserve further investigation, all data available so far clearly argue against the previously suggested prophylactic MBL replacement in HSCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Bone Marrow Transplantation website (http://www.nature.com/bmt)

SUPPLEMENTARY INFORMATION

Patients

In total, ninety-nine pediatric and young adult patients, median age 8.8 years (range 1.4 – 24 years) consecutively undergoing allogeneic HSCT at the transplantation unit of the St. Anna Children's Hospital, Vienna, Austria, were enrolled between June 2007 and March 2012. The study was approved by the Institutional Review Board and was performed according to the guidelines of the Helsinki Declaration of Human Rights.

Diagnoses and HSCT conditions: The diagnoses and specific conditions of HSCT in the present cohort are summarized in supplementary table 1.

Supplementary Table 1. Clinical features and conditions of HSCT

	conditions of HSCT	gender		
		female	**male**	**total**
diagnoses	leukemia/lymphoma	17	45	62
	hematolologic diseases	5	9	14
	primary immunodeficiencies	7	7	14
	solid tumors	3	6	9
conditioning regimen	myeloablative	18	50	68
	with TBI	10	33	43
	reduced intensity	14	17	31
	serotherapy* in conditioning	26	55	81
stem cell donors	matched** unrelated	13	45	58
	matched sibling	12	19	31
	mismatched family	7	3	10
stem cell sources	bone marrow	19	54	73
	peripheral blood	12	12	24
	bone marrow + peripheral blood	1	0	1
	umbilical cord blood + bone marrow	0	1	1

Supplementary Table 1: TBI, total body irradiation; * Serotherapy used within the conditioning regimen included anti-thymocyte globulin (n = 66), OKT3 (n = 10), and alemtuzumab (n = 2); 2 patients received a combination of ATG and alemtuzumab, 19 patients did not receive any serotherapy as part of the conditioning.

** High resolution HLA genotyping was performed at the Department of Blood Group Serology and Transfusion Medicine/Medical University Vienna, Austria. Matched unrelated and matched sibling donors were defined by matching 10 of 10 HLA genotypes. Mismatched family donors were haploidentical parents.

GVHD: After HSCT, all patients received cyclosporine with or without methotrexate or mycophenolate-mofetil for GVHD prophylaxis. Tacrolimus was used when cyclosporine was not tolerated. In case of GVHD, patients routinely received steroid treatment as a first line treatment. Non-responding patients received individualized immunosuppressive treatment combinations.

MBL analyses:

MBL genotyping: Genomic DNA was extracted from cell pellets ($5x10^6$ nucleated cells) using the QIAamp® DNA Mini Kit (50) (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. A high resolution melting analysis (HRMA) was adapted for detection of MBL exon-1 SNPs B, C and D and the promoter variants H/L, and Y/X. Samples identified by HRMA to harbor exon-1 SNPs were further analyzed by sequencing (VBC-Biotech Service GmbH, Vienna, Austria).

MBL serum level determination: the MBL Oligomer ELISA Kit (Bioporto Diagnostics, Gentofte, Denmark) was used and MASP-2 levels (performed in 83 patients) were quantified using the MASP-2 ELISA Kit HK326 (Hycult Biotech, Uden, The Netherlands) according to the manufacturer's instructions. Because of insufficient sample collection, MBL genotypes

and MBL and MASP-2 serum levels were successfully determined in 96 and 88 and 83 patients, respectively.

Supplementary Table 2. MBL2 primers for sequencing and HRMA analysis

MBL2 sequencing primer			
promoter (fw)	AATGGGAGGAGGATTCAAGG	**promoter (rev)**	AGGCACTATGATGAGCAGTGG
exon 1 (fw)	ACGCAGTGTCACAAGGAATG	**exon 1 (rev)**	ATCAGTCTCCTCATATCCCCAG
MBL2 HRMA primer			
-619 (fw)	GTAGTAAGAAATTTCCAGAGAAAATGC	**-619 (rev)**	AGTTTGCTTCCCCTTGGTG
-290 (fw)	CATTCCCTAAGCTAACAGGCATAAG	**-290 (rev)**	TAGACACCTGGCGTTGCTG
exon 1 (fw)	CCTGTAGCTCTCCAGGCATC	**exon 1 (rev)**	AGAGACAGAACAGCCCAACAC

Supplementary Table 2: The bold columns define investigated region in the MBL 2 gene, with 'fw' indicating forward and 'rev' indicating reverse primer; Promoter regions -619 and -290 define the position of the variation as the distance from the transcriptional start site (ATG); HRMA primer were used for the high resolution melting process, sequencing primer for sequencing HRMA-identified heterozygous DNA samples. Reference sequence for genomic DNA: NG_008196.1

MBL genotypes and MBL and MASP-2 serum levels

Of the 96 patients examined for MBL genotypes, 60 were homozygous A/A and 33 harbored a heterozygous exon 1 SNP. Three patients carried a homozygous exon 1 polymorphism (BD, CC, BB). Of 88 patients in whom MBL serum levels were determined, 12 had MBL serum levels ≤ 300 ng/mL, 9 of them carried a heterozygous 'A/0' genotype. In patients with homozygous '0' genotypes the MBL serum levels were very low (CC, 4.6 ng/mL; BD, 17 ng/mL; and BB, 30 ng/mL). According to their expected serum levels, MBL genotypes were divided in 4 groups, high, medium, low and very low (Supplementary Table 3). Overall, this classification system proved useful for a statistically significant genotype-phenotype correlation ($p < 0{,}001$) (Supplementary Figure 1). The following considerations have been

taken into account: Firstly, in contrast to the „B□ and □C□ variant, patients with an A/D genotype commonly have higher MBL levels and function[4]. Secondly, homozygous „LXA□ haplotypes are associated with moderately reduced MBL serum levels and their function is comparable with heterozygous exon 1 mutations. Thus, patients with an LXA/LXA genotype were allocated to the "medium" group. MASP-2 serum levels (not shown) ranged from 244 – 2512 ng/mL, thus, no patient with MASP-2 deficiency was identified in this study.

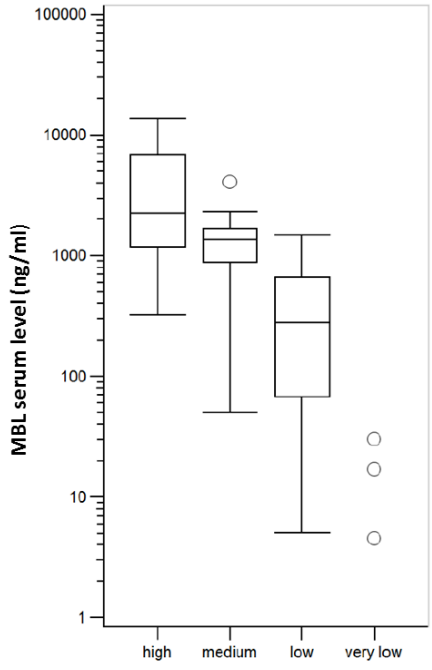
Supplementary Table 3.

Classification system correlating MBL genotype to expected serum levels

HIGH	
homozygous A without LX/LX	HYA/HYA
	HYA/LYA
	LYA/LYA
	HYA/LXA
	LYA/LXA
heterozygous D on H or LY background	HYA/HYD
	LYA/HYD
MEDIUM	
LX/LX	LXA/LXA
heterozygous D on LX background	LXA/HYD
heterozygous B or C on H background	LYB/HYA
	LYC/HYA
LOW	
homozygous D	HYD/HYD
heterozygous B or C on L background	LYB/LXA
	LYB/LYA
	LYC/LXA
	LYC/LYA
VERY LOW	
Homozygous B or C	LYB/LYB
	LYC/LYC
Compound heterozygous B and C	LYB/LYC
Compound heterozygous B or C on D background	LYB/HYD
	LYC/HYD

Supplementary Table 3: The left column indicates the classification system of MBL exon 1 variants (A,B,C,D) referred to promoter dimorphisms (H/L, Y/X) on the corresponding allele according to expected high, medium, low or very low MBL serum levels. The right column indicates the full genotypes in the respective group, the 6 investigated common haplotypes (HYA, LYA, LXA, HYD, LYC, LYB) giving 21 haplotype combinations.

Supplementary Figure 1



N	50	16	16	3
Median	2264	1360	279	4.6, 17, 30
min-max	323 - 13840	50 - 4975	5 - 1480	
p-value	<0.001			

Supplementary Figure 1. Correlation of MBL genotypes and the range of MBL serum levels.

The box plots depict the correlation of the MBL serum levels and MBL genotypes according to the classification system as proposed in supplementary table 3. The boxes indicate the 25th to 75th percentiles and the lines indicate the range. The horizontal lines within the boxes indicate the median. The circles depict the individual MBL serum levels in the three cases with homozygous exon 1 '0' genotpyes. The statistical analysis demonstrates that despite significant overlap, the genotype classification largely correlates with the range of actual serum levels.

Statistical analysis

Probability of 2-years overall survival (2-yrs. pSU) was estimated according to the method of Kaplan-Meier. Confidence intervals were calculated based on log-log transformation of standard errors according to Greenwoods[1]. For 2-yrs. pSU, deaths of any cause were considered an event. Patients without event were censored at the date of last follow-up. The statistical comparison of 2-yrs. pSU of patient groups was done by using the log-rank test. The cumulative incidences of transplantation related mortality and deaths of disease were estimated taking into account these competing risks[2]. A cut-off level of MBL $\leq$ 300 ng/mL [3,4] was used to investigate the impact of MBL deficiency in HSCT. For comparison of categorical outcomes (e.g. acute GVHD, or events as listed in table 1 of the full text), Fisher's exact and Chi-Square tests were used[5]. The impact of variables showing statistical significance in univariate analysis for estimating the GVHD risk was further investigated in multivariate analyses by logistic regression[6] and is given as the odd's ratio.

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