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Olaf-Werner Neth, Ulrike Bacher, Purnima Das, Tatjana Zabelina, Hartmut Kabisch, et al.. Influence of mannose-binding lectin genotypes and serostatus in allo-SCT: analysis of 131 recipients and donors.. Bone Marrow Transplantation, Nature Publishing Group, 2010, 45 (1), pp.13-9. <10.1038/bmt.2009.90>. <inserm-00414612>

HAL Id: inserm-00414612

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Submitted on 17 May 2010

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Influence of Mannose Binding Lectin (MBL) Genotypes and Serostatus in Allogeneic Stem Cell Transplantation: Analysis of 131 Recipients and Donors

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Running title: Influence of Mannose binding lectin after transplantation

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Summary

Background: Mannan-binding lectin (MBL) deficiency is determined by *MBL* gene polymorphisms and associated with an increased infection risk. **Methods:** To clarify the role of MBL in allogeneic stem cell transplantation (SCT) 131 recipients/donors were analysed. *MBL*-genotypes were determined by PCR and heteroduplex analyses, MBL-serum levels by ELISA, MBL oligomers by Western blotting. **Results:** MBL-levels <400ng/ml were associated with increased susceptibility to fungal pneumonia (7/12 vs. 35/111; $p=0.04$, adjusted $p=0.002$), HSV/VZV (7/12 vs. 26/111; $p=0.03$), CMV-reactivation and acute graft-versus-host-disease. Donor genotypes had no influence. Pre-SCT MBL-levels corresponded to recipients' genotypes ($p<0.001$), changed significantly post-SCT, but were not influenced by donors' genotypes. MBL oligomer profiles were similar pre-/post-SCT. Cultured CD34+ cells were not found to synthesize MBL. **Conclusions:** Low MBL-levels pre-transplant predisposed patients to sepsis, fungal and viral infection. Donors' MBL genotypes did not influence infection rates. Prospective studies should clarify the importance of MBL as prelude for MBL-replacement after SCT.

Key words: Mannan-binding lectin (MBL) protein, MBL variants, sepsis, infection, allogeneic stem cell transplantation (SCT).

Introduction

Mannan-binding lectin (MBL) is a component of the innate immune system. The carbohydrate recognition domain of the MBL protein binds to polysaccharides of micro-organisms and activates the complement system in an antibody independent fashion.¹ Mutations in the structural and regulatory sequences of the MBL gene lead to great inter-individual variation.² MBL variants due to single nucleotide polymorphisms in exon 1 - B (G54D), C (G57E), and D (R52C)^{1:3} - are found in approximately 30% of the general population.

Several studies have demonstrated increased infection rates in patients with low MBL levels^{4:5} and also in neutropenia following chemotherapy.^{6:7} Recently, *MBL* variants were associated with an increased risk of infections in myeloablative, TBI-conditioned transplantation.⁸ Conversely, patients with wildtype genotypes have a reduced sepsis risk during autologous stem cell transplantation (SCT).⁹ Limited studies show an influence of both recipients and donor genotypes¹⁰⁻¹² on the infection risk in this setting. In solid organ recipients, MBL deficiency is associated with increased CMV reactivation.¹¹ MBL may also influence the development of graft *versus* host disease (GvHD) and graft rejection in solid organ recipients.¹³

We analysed 131 stem cell recipients to evaluate the influence of MBL on transplant related mortality (TRM), infection, and acute GvHD (aGvHD) in the allo-SCT setting.

Patients and Methods

Patients

From January 2001 - October 2003 a total of 131 patients, who received allo-SCT from related/unrelated donors at the University of Hamburg, Germany, were enrolled in a combined pro-/retrospective study after ethical approval and patients' informed consent.

***MBL* genotype and protein analyses**

Blood for MBL pheno- and genotypes was collected pre-, on days 0-45 and days +46-75 post-transplant. MBL serum levels were determined by ELISA (Antibody Shop, Denmark). DNA was isolated from whole-blood samples (QIAamp DNA, blood mini kit, Qiagen, Hilden, Germany).

The *MBL* variants in Exon 1 B, C and D (codons 54, 57, and 52) were determined by PCR and heteroduplex analyses utilising polyacrylamide gel electrophoresis.¹⁴ Patients homozygous for wildtype alleles were denoted A/A, homozygous for variant alleles O/O, and heterozygous A/O. Promoter polymorphisms of *MBL* were determined similarly.¹⁵ The structural *MBL-2* mutations B, C, and D are in linkage disequilibrium with the promoter region

polymorphism X/Y, so only Y associates with variant alleles. Western blot analyses of MBL protein pre- and post-transplant were performed using SDS-Page gel (Hybond C extra, Amersham Biosciences, UK) and immunoblotting as described previously.¹⁶ To investigate whether MBL is synthesized by CD34⁺ cells, mononuclear cells were obtained from three bone marrow samples (donors with *MBL* wildtype) and CD34⁺ cells were separated and cultured as previous described.¹⁷ MBL concentrations in cultures were analysed on day 14 using the ELISA.

Infection, GVHD and TRM

Patients were monitored during the study period (days 0 to +75) for frequency of infections, GVHD and TRM. Blood cultures, C-reactive protein (CRP) and imaging were performed when infection was suspected. Definite sepsis was diagnosed in case of positive blood cultures and clinical and/or laboratory sepsis evidence. Clinical sepsis was diagnosed in absence of a positive blood culture. Diagnosis of sepsis was retrospectively made by two blinded clinicians. Viral reactivation, GVHD and TRM were also recorded. Fungal pneumonia was defined as presumed, possible or definitive.¹⁸

Statistical analysis

Patients were classified according to MBL exon-1 mutations and their corresponding promoter allotypes as follows: Patients with YA/YA, YA/XA, or XA/XA genotypes were wildtype (A/A), patients with YA/YD, YA/ YB+YC, XA/YD or XA/YB+YC heterozygous (A/O), and patients with YO/YO MBL genotype homozygous (O/O).

Genotype/allele frequencies were analysed by Chi-square. Differences between groups (MBL serum levels) were compared by Mann-Whitney U Test. Changes in mean MBL values were performed by Wilcoxon's signed rank test. The analyses of the risk of sepsis, viral reactivation, GVHD, and TRM according to MBL genotype and serum levels were performed by log-rank test and Kaplan-Meier. In addition, multivariable analysis (Cox proportional hazard model) was performed to estimate the independent effect of MBL level on fungal pneumonia, CMV-reactivation and acute graft-versus-host-disease adjusted for recipient and donor age, sex, diagnosis, type of transplant (BM vs. PBSC), type of donor (unrelated or related), HLA type (match or mismatch), conditioning (standard or reduced), type of GvHD prophylaxis (with or without antithymocyte globulin), MBL genotypes of recipient and donor. SPSS version 13.0 (SPSS, Inc, Chicago, IL, USA) and SAS v9.1 (SAS®, Cary, NC, USA) was used for analyses.

Results

Patients' and donors' characteristics

Characteristics of donor and recipients are summarised in *Table 1*.

Analysis of *MBL* genotypes

MBL-2 genotypes were successfully determined in 130 patients and donors. Donors' and recipients' genotypes were similarly distributed and corresponded to previous data (*Table 2*).^{4;9;19}

Infection

Fifty-six patients suffered from one clinical septic episode or more. Definite (blood culture positive, most frequently with gram-positive organisms) sepsis was observed in 50 patients (n=72). There were 18 gram negative septic episodes and seven patients developed fungal sepsis. The first episode occurred between days 0 and +252 post-transplant (median 52). Over 50% of patients suffered from pneumonia, a third from CMV reactivation, and a quarter from VZV/HSV reactivation (*Table 1*).

MBL genotype and infection

There was no significant association between *MBL* genotypes and sepsis. 37/87 *MBL* wildtype patients developed bacterial sepsis compared to 13/43 patients with variant alleles (p=0.32). No association was observed between recipients' *MBL* genotypes and pneumonia (p=0.26) or CMV reactivation (p=0.96). VZV/HSV reactivation was higher in patients with variant alleles (15/43 compared to 18/87 patients with *MBL* wildtype, p=0.08). Donor *MBL* genotype was not associated with any above parameter.

MBL genotypes of recipients and donors did not influence the severity of aGVHD (p=0.76 and 0.28) or TRM (p=0.44 and 0.26).

MBL phenotype analysis

Pre-SCT *MBL* levels (available from 123 patients) were significantly related to *MBL-2* genotypes (p < 0.001) (*Figure 1*). In 29/87 A/A genotype patients, where *MBL* levels were available pre-SCT, on days 0-45 and +46-75 (*Figure 2a*), *MBL* levels increased significantly from a mean of 3580 ng/ml pre-transplant to 5767 ng/ml by day +45 (p<0.001). Levels declined over the next 30 days to a mean of 4136 ng/ml (p=0.15). **In a robustness analysis excluding two patients of this group because of extreme values, results were similar (3000 ng/ml to 4343 ng/ml by day +45; p=0.02 and 3758 ng/ml by day 75; p=0.20).**

In 19/43 A/O and O/O patients, samples were available for all three time points. *MBL* levels increased from a mean of 1021 ng/ml pre-transplant to 1476 ng/ml by day +45 (p=0.32) and

fell to a mean of 594 ng/ml by day 46-75 ($p=0.21$, *Figure 2a*). If YA/YD individuals (whose levels are frequently in the same range as in A/A) were included in the wildtype group, the pattern remained similar (*Figure 2b*).

To investigate whether MBL levels were influenced by donor derived cells, levels were measured in 14 variant patients (A/O or O/O) with wildtype donors. Levels did not change significantly pre- to post-transplant ($p=0.26$) and did not alter in wildtype recipients and variant donors ($n=11$, $p=0.92$).

To explore MBL expression following SCT, Western blot analysis was performed from a YA/YD recipient who received SCT from a XA/XA donor. MBL concentrations pre-transplant were 843 ng/ml and 993 ng/ml post-transplant. MBL protein profiles pre- and post-SCT were similar, and did not increase in high order oligomers as seen in WT individuals (*Figure 3*). Finally, MBL was not detectable from CD34⁺ cells cultured from three donor marrows with wildtype MBL genotype.

MBL phenotype and infection

Sepsis rates were not significantly associated with MBL levels $<400\text{ng/ml}$ ($p=0.57$), but MBL levels in the first or last quartile (low and high MBL levels, $790 \geq \text{MBL} > 3418 \text{ ng/ml}$) showed an increased risk of culture proven sepsis ($p=0.05$) and a trend towards clinical sepsis ($p=0.07$) and fungal pneumonia ($p=0.06$) when compared with the 2nd/3rd quartiles (normal MBL levels, $790 < \text{MBL} \leq 3418 \text{ ng/ml}$). Twelve patients had levels of $<400\text{ng/ml}$ (lowest 10%) and suffered significantly more from fungal pneumonia (7/12 vs. 35/111; $p=0.04$), HSV/VZV infection/reactivation (7/12 vs. 26/111; $p=0.03$), and also CMV infection/reactivation was higher (6/12; 50% vs. 36/111; 32%; n.s., *Figure 4a-c*). When multivariable analysis was performed, the risk of fungal pneumonia was significantly higher in male patients (HR= 2.9, $p=0.008$) and in patients with serum MBL levels $\leq 400 \text{ ng/ml}$ (HR= 4.1, $p=0.002$). MBL was the only one factor associated with risk of sepsis, HSV/VZV and CMV infection/reactivation.

MBL phenotype and risks of GVHD and TRM

There was no significant association between MBL levels and TRM or acute GVHD. However 7/12 patients with MBL levels $<400 \text{ ng/ml}$ suffered from aGVHD compared to 37/111 with MBL levels $\geq 400 \text{ ng/ml}$ (58% versus 33%, *Figure 4d*), half of the patients with MBL levels $<400 \text{ ng/ml}$ suffered from TRM compared to 31 out of 111 (50% versus 28%).

Discussion

Infections still contribute to TRM in recipients of allo-SCT. Previous studies suggested an influence of MBL status on infection in neutropenic patients.^{6,7} We analysed 131 stem cell recipients to clarify the role of donors' and recipients' MBL genotypes and MBL serum levels on infectious and transplant related complications.

MBL-genotype did not significantly influence infections, GvHD, or TRM in this study, whereas previous studies found an association between MBL genotype and transplant-related complications.^{8,10} However, we found an association between low MBL levels and fungal pneumonia, HSV/VZV infection and observed a relationship between sepsis and low and high MBL levels. Thus, MBL levels may be more important than genotypes in this setting.

MBL serum levels increased significantly following SCT before returning to pre-transplant levels by day +75. Overall the mean MBL levels pre-transplant were elevated to twice those in healthy populations which may reflect the pro-inflammatory state of patients undergoing SCT.¹

We investigated the hypothesis that donor genotypes influence MBL levels.^{10,15} Levels did not significantly change in variant patients (A/O or O/O) with WT donors. Also, MBL levels did not alter in WT recipients who received SCT from variant donors, and donor genotypes did not influence the risk of transplant-related complications. We also failed to detect MBL in cultured CD34⁺ stem cells or a change to the oligomeric MBL profile in a patient with a heterozygous MBL genotype who received a wildtype donor SCT. Our results therefore support the study of Kilpatrick *et al.* where there was no effect of donor genotype on circulating MBL levels in two patients receiving SCT.²⁰ In contrast, **after** liver transplantation, the genotype of the donor confers the subsequent MBL phenotype. Donations of livers (the main MBL source) from variant donors to wildtype recipients resulted in rapidly decreasing MBL serum levels.²¹ This keeps with previous studies²² where donors' genotypes did not influence transplant-related complications. Therefore, it is unclear how to explain the findings of Mullighan *et al.* and Granell *et al.* that transplantation outcome depended on MBL genotypes of both recipients and donors.^{10,12}

The observations from this study that the only clinically significant associations were related to MBL levels indicate that phenotype may be more important than genotype in predicting susceptibility to infection. 58% of patients with low MBL levels developed presumed fungal pneumonia and HSV/VZV reactivation, as compared to 31% and 23% in patients with pre-SCT levels >400ng/ml. Two patients with wildtype genotype and MBL levels <400ng/ml developed sepsis and fungal pneumonia. The low levels might be related to pre-transplant clinical status. **Surprisingly**, two homozygous O/O patients had MBL serum levels >400ng/ml **although there is normally a strong correlation between MBL genotype and phenotype. One hypothesis might be** repeated erythrocyte transfusions from donors with higher MBL **but this alone is unlikely to fully explain these findings. The MBL levels of these two patients decreased post-transplant.** It should be noticed that the only other independent predictor of susceptibility of infection was male **sex**. This result is not explained by current biological knowledge, and therefore remains to be confirmed in other studies. Also, a selection bias that could have generated this spurious association might be discussed.

MBL deficiency has emerged as a risk factor for mycosis probably due to the presence of MBL ligands such as mannan on various fungi.²³ We also observed a relationship to HSV/VZV reactivation.

There is considerable debate about the definition of low MBL levels. Previous studies indicated that susceptibility to infection may vary at different MBL levels depending upon clinical circumstances and patient age. The use of different assays might also contribute to MBL level variability.²⁴ Previously we found that MBL levels <400ng/ml did not activate complement following incubation with bacteria and failed to enhance MBL mediated opsonophagocytosis.²⁵ Levels \leq 400ng/ml correlated highly with YO/YO, XA/YB, or XA/YC genotypes in 500 healthy children (own unpublished observations).

Our data correlate with previous reports demonstrating an association of low MBL levels and increased infection rates in haematological patients with neutropenia^{6,7} and of a protective role of WT MBL genotype during autologous SCT.⁹ Additionally, we observed an increased rate of sepsis, not only in patients with low MBL levels but also in patients with very high levels. Schlapbach *et al.* saw similar results in a study of 94 paediatric cancer patients with treatment-associated neutropenia.⁷

Why high MBL levels should predispose patients to sepsis remains unclear. MBL might influence the host inflammatory response to invading microorganisms. In *ex vivo* experiments, MBL levels had a dose-dependent effect on the inflammatory response to *Neisseria meningitides*,²⁶ and MBL influenced anti-inflammatory cytokines.²⁷ The balance between pro- and anti-inflammatory responses has been shown to influence the susceptibility to sepsis,²⁸ so high and low MBL levels might increase sepsis rates. High MBL levels may also merely reflect the inflammatory response to infection.

In conclusion, low MBL levels predispose patients undergoing SCT to sepsis, fungal pneumonia, and HSV/VZV infection. Larger prospective studies should fully evaluate the effect of MBL status after SCT and the potential role of MBL replacement therapy to prevent infection in this clinical setting.

Acknowledgments: We would like to thank the staff of the Infectious Diseases and Microbiology Unit and for assistance with the MBL assays. RT was supported by a grant from the Agence nationale de recherches sur le sida et les hépatites virales (ANRS).

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Table 1: Patient/Donor characteristics, complications and outcome (n=131)

PATIENT	
Age at SCT in years (median)	13-69 (46)
Sex of patients (m/f)	83/48
CMV positive	75 (58%)
DONOR	
Sex of donors (m/f)	90/41
CMV positive	73 (56%)
Matched/Mismatched	107/24
Related/Unrelated	51/80
DISEASE	
AML	35 (27%)
ALL	16 (12%)
Lymphoma	11 (8%)
Multiple Myeloma	20 (15%)
CML	26 (20%)
Myelodysplastic Syndrome	10 (8%)
Others	13 (10%)
TYPE OF TRANSPLANT	
PBSC	109 (83%)
BMT	22 (17%)
CONDITIONING REGIMENS	
Bu/Cy+/-VP16	50 (38%)
TBI/Cy/VP16	19 (14%)
RIC	62 (48%)
ENGRAFTMENT	
Leukocyte ($\geq 1.0 \times 10^9/L$)	6-26 days (mean 15)
Lymphocyte ($\times 10^6/ml$)	20-3340 (mean 1008)
NK cells ($\times 10^6/ml$)	8-858 (mean 239)
ACUTE GVHD	
Grade 0-I	82 (63%)
Grade II-IV	49 (37%)
Grade III-IV	29 (22%)
INFECTIONS	
Definite sepsis	50 (38%)
Clinical sepsis	56 (43%)
Pneumonia	76 (58%)
Fungal	46 (35%)
Bacterial	17 (13%)
Atypical	8 (6%)
Viral	5 (4%)
CMV infection/reactivation	
Yes	46 (35%)
No	85 (65%)
VZV/HSV infection/reactivation	
Yes	33 (25%)
No	98 (75%)
DEATH AND CAUSE	n=62 (47%)
Infection	23 (17.5%)
TRM	37 (28%)
Relapse	20
GvHD	8
GvHD with infection	6

Toxicity Other	3 2 (1.5%)
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PBCST, peripheral blood stem cell transplantation; *BMT*, bone marrow transplantation; *NK* cells, natural killer cells; *Bu*, Busulfan; *Cy* cyclophosphamide; *VP16*, Etoposide; *TBI*, total body irradiation; *RIC*, reduced intensification conditioning

Table 2: Frequency of MBL Exon-1 and X/Y promoter polymorphisms

MBL Genotype	Recipient n (%)	Donor n (%)
YA/ YA	48 (36.6)	46 (35.1)
YA/ XA	30 (22.9)	32 (24.4)
XA/ XA	9 (6.9)	6 (4.6)
Wildtype (A/A)	87 (66.4)	84 (64.1)
YA/YD	12 (9.2)	17 (13.0)
YA/ YB+YC	20 (15.2)	15 (11.5)
XA/YD	3 (2.3)	2 (1.5)
XA/ YB+YC	3 (2.3)	2 (1.5)
Heterozygous (A/O)	38 (29.0)	36 (27.5)
Homozygous (O/O)	5 (3.8)	10 (7.6)
Missing	1 (0.8)	1 (0.8)
Total	131 (100)	131 (100)

Patients with either YA/ YA, YA/ XA, XA/ XA or genotype were summarized as A/A; YA/YD, YA/ YB+YC, XA/YD or XA/ YB+YC patients were defined as A/O and patients with YO/YO MBL genotype as O/O.

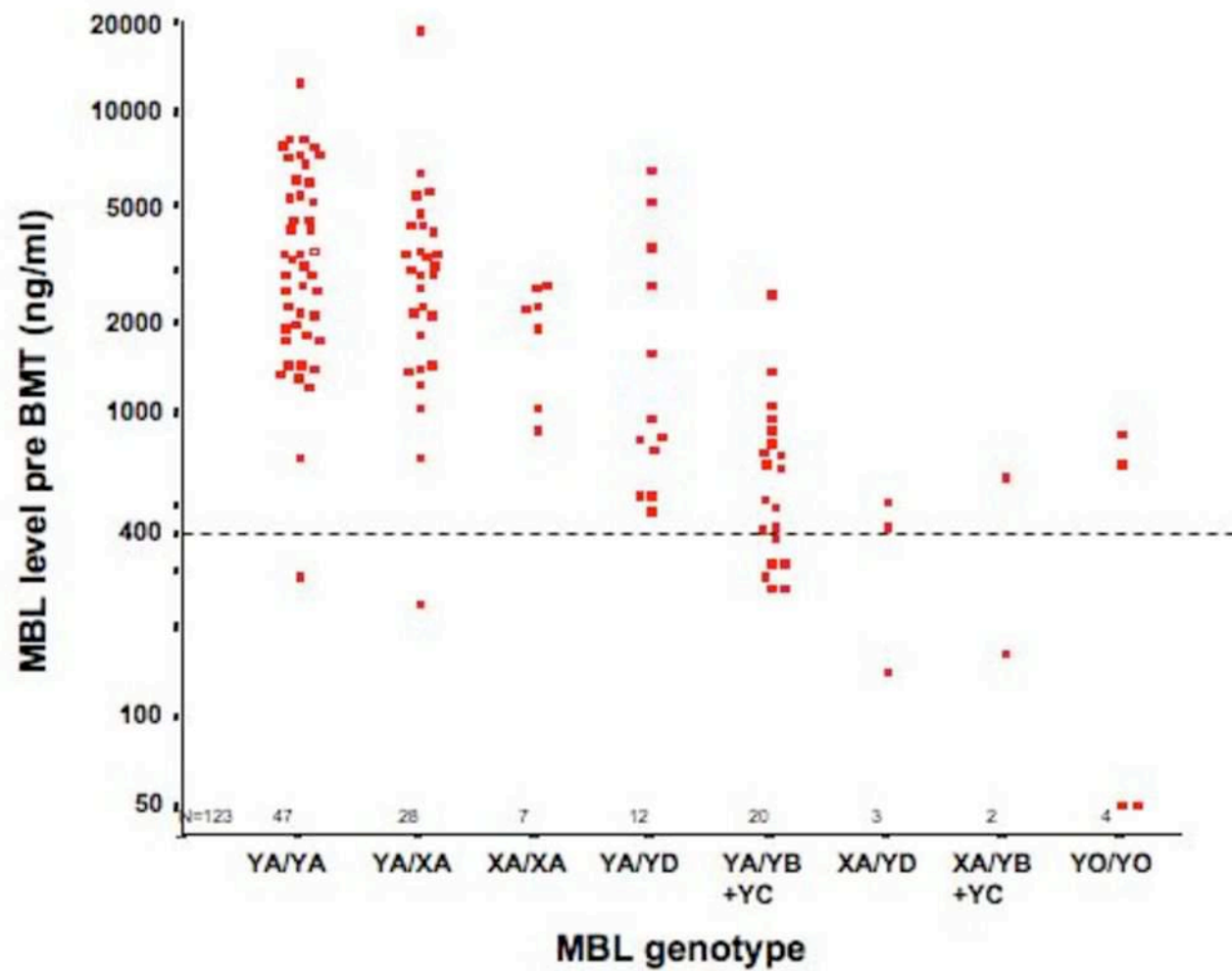


Figure 1

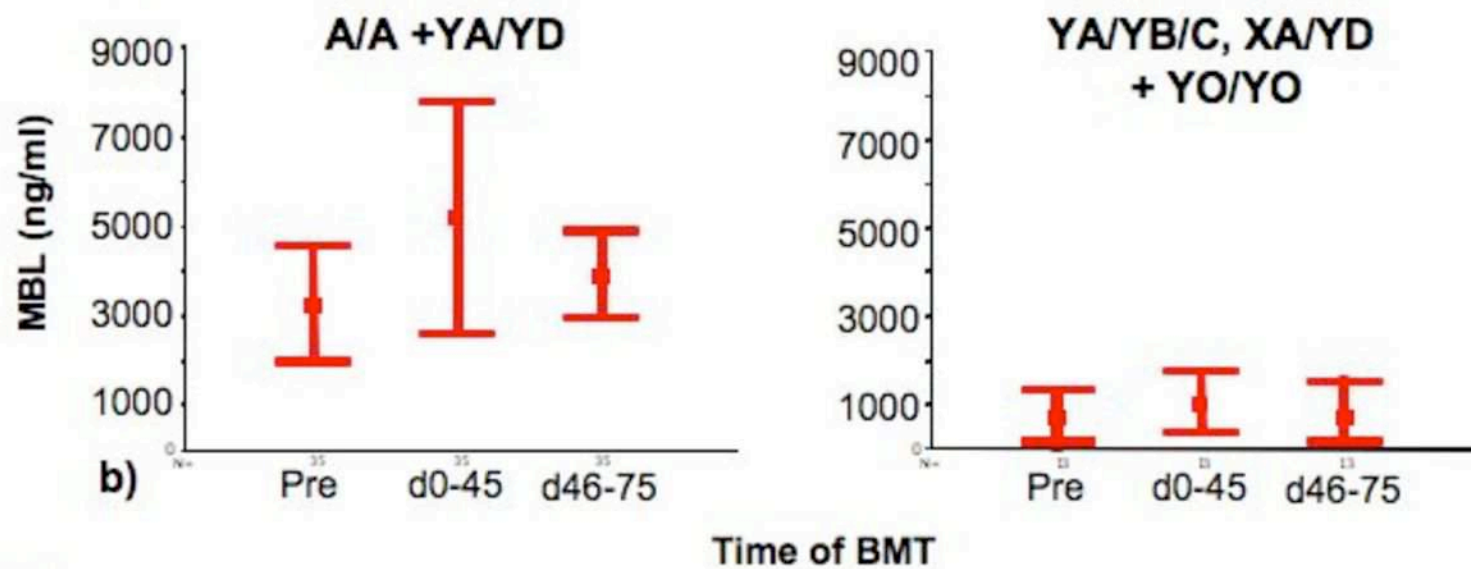
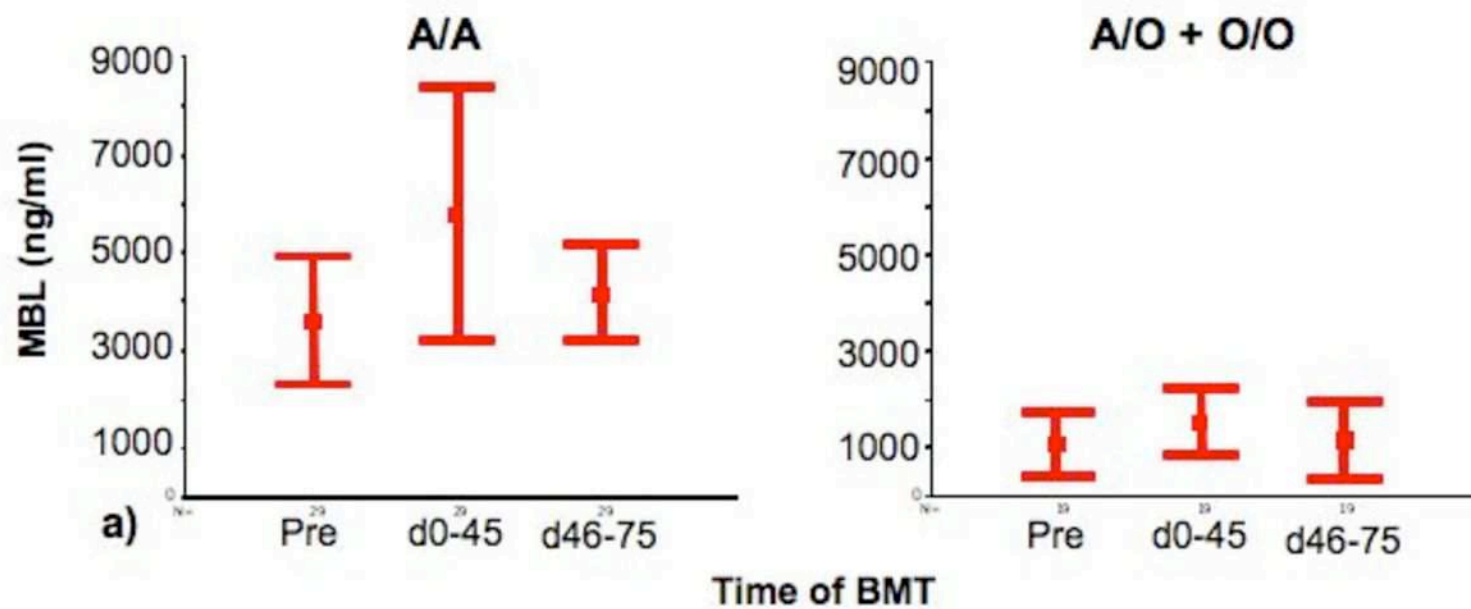


Figure 2

**MBL protein expression pre- and post BMT
by Western blot analyses**

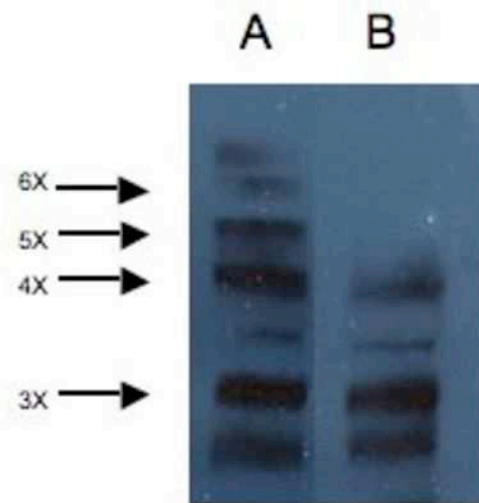
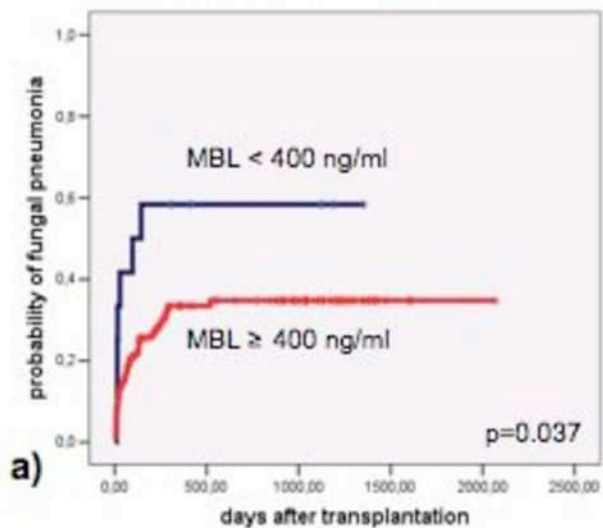
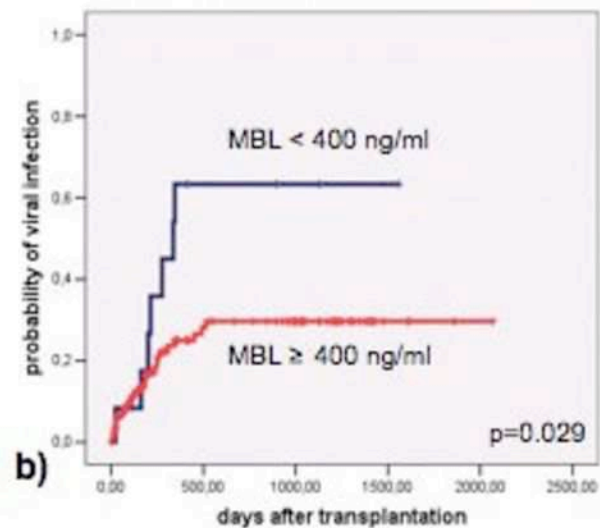


Figure 3

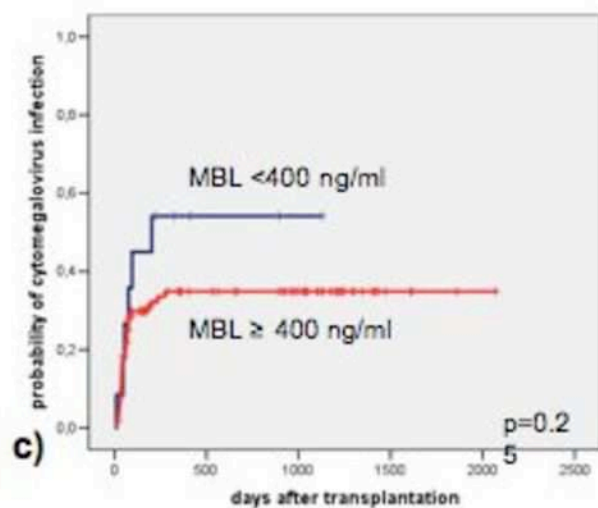
Fungal Pneumonia



HSV/VZV Infection/Reactivation



CMV Infection/Reactivation



Acute GvHD

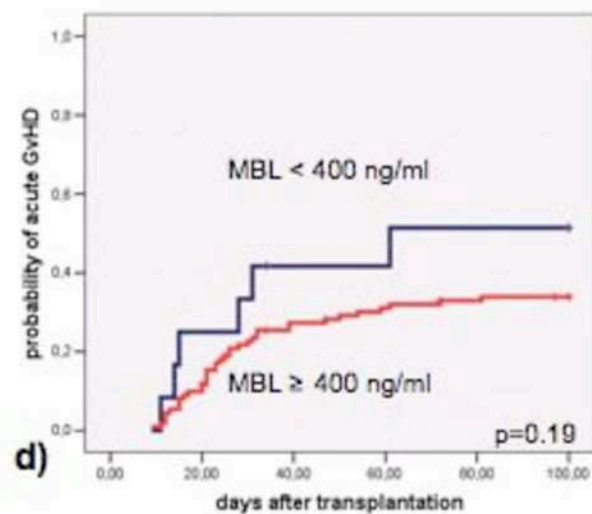


Figure 4

Figure Legend

Figure 1: Correlation of MBL genotype and phenotype pre SCT.

The horizontal dashed line represents MBL levels of 400ng/ml.

Figure 2: Serial measurements of MBL serum levels pre and post SCT in patients with a) wild type MBL genotype (A/A) and heterozygous (A/O) or homozygous (O/O) MBL genotype and b) with A/A + YA/YD and YA/YB/C, XA/YD + YO/YO MBL genotype. Levels are expressed as Mean +/-2SE.

Figure 3:

MBL protein expression pre- and post SCT by Western blot analyses.

MBL tetramers and pentamers are expressed as x4 and x5 respectively. MBL protein pre SCT is seen in lane A and post BMT in lane B. Correspondent MBL concentration pre SCT was 843 ng/ml and 993 ng/ml post SCT. MBL genotype of recipient was YA/YD (A/O) and of the donor were XA/XA (A/A).

Figure 4:

Association of very low MBL serum levels (<400ng/ml) with infection and acute GvHD.