The role of mannose-binding lectin in vitro and in vivo
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CHAPTER 10

Summary and general discussion
Mannose-binding lectin (MBL) is a component of the innate immune system that activates the complement system upon binding to repeating sugar residues on the surface of microorganisms or altered host cells. This first line of defense against micro-organisms is of importance to prevent or clear infections in young children who have not yet developed their immunoglobulin repertoire, and in later life during the first days after infection with a new micro-organism before the adaptive immune response has been induced. MBL-mediated activation of the lectin pathway of the complement system leads to opsonization of invading micro-organisms (C3b and iC3b deposition) and promotes their phagocytosis and thereby the clearance of micro-organisms from the body. Besides opsonization, complement activation also promotes chemotaxis (C5a) and lysis of the micro-organisms (C5b-9, MAC). MBL2 variant alleles lead to reduced or deficient levels of MBL in the circulation and are very common (about 30% of the Caucasian population carries a variant allele). MBL deficiency might cause an increased risk of infections or infectious complications concomitant with other disease.

In this thesis the role of MBL in complement activation and opsonophagocytosis \textit{in vitro} (chapter 2 and 3) and MBL deficiency as a risk factor for clinical complications \textit{in vivo} (chapter 4-7) is discussed. The last two chapters (chapter 8 and 9) focus on the potential of plasma-purified MBL as a therapeutic agent.

\textit{In vitro} MBL-mediated complement activation and opsonophagocytosis.

To study the MBL-mediated opsonization and phagocytosis of different micro-organisms, we developed an \textit{in vitro} flow cytometry assay. Micro-organisms were opsonized with serum from either MBL-sufficient or MBL-deficient donors, after which the opsonized micro-organisms were phagocytized by isolated human neutrophils from an unrelated healthy donor. By the use of this assay we found that zymosan particles (\textit{Saccharomyces cerevisiae} cell-wall fragments) are mainly opsonized via the lectin pathway of complement activation. Opsonization of zymosan with MBL-deficient serum led to a 3-fold reduction of phagocytosis compared to zymosan opsonized with MBL-sufficient serum. We further found that the lectin pathway-mediated opsonization of zymosan was enhanced by the alternative pathway amplification loop, even at low (3% v/v) serum concentrations. Opsonophagocytosis of zymosan opsonized with MBL-deficient serum or combined MBL- and alternative pathway-deficient serum was restored by addition of purified MBL or purified alternative pathway proteins to the opsonization step. In conclusion, the C4b2a-complex, formed upon activation of the lectin pathway, and the C3bBb-complex, formed by activation of the amplification loop of the alternative pathway, act together for optimal C3 convertase activity and optimal MBL-mediated opsonization of zymosan.

The binding of MBL to various micro-organisms (gram+ / gram- bacteria, fungi, viruses and protozoa) has been widely studied, but usually in the absence of a complement source (serum /
plasma), whereas we showed that the lectin pathway-mediated opsonization is highly amplified by the alternative pathway. Furthermore, MBL binding alone is not sufficient for optimal opsonization, because complement activation is essential as well. We found that although MBL binds to various bacterial strains, the role of attached MBL in complement-mediated opsonization of these bacteria is limited. The bacterial strains were opsonized mainly via the classical pathway of complement (antibody-mediated), and this opsonization was also highly amplified by the alternative pathway. This is in contrast to yeast opsonization, where MBL binding and subsequent complement activation strongly enhanced phagocytosis of the opsonized yeasts.

The discrepancy between MBL binding to bacteria and MBL-mediated phagocytosis of these bacteria suggest another role for MBL-binding to bacteria. Binding of MBL-C3b/d complexes to complement receptor-1 present on the surface of neutrophils has been described (1). It has also been suggested that there are specific collectin receptors on the surface of phagocytes (2). These receptors could provide a means for MBL-IgG-mediated phagocytosis of opsonized bacteria without contribution of the complement system. MBL binding to micro-organisms may induce other innate (or adaptive) immune systems, besides the complement system, which may contribute to clearance of the micro-organisms from the circulation. Cytokines are signaling proteins used in cellular communication and are produced by macrophages, monocytes and dendritic cells. Several studies have reported a role for MBL in cytokine production and secretion. Most reports describe an increase in the anti-inflammatory cytokines IL-6, IL-8, IL-10 and RANTES (5), and a suppression of the pro-inflammatory cytokines IL-1α, IL-1β, TNFα (3, 4). These data suggest that MBL bound to bacteria may enhance phagocyte recruitment to the site of infection, while down-regulating macrophage-mediated inflammation in a complement-independent manner.

A disadvantage of the in vitro MBL binding and complement activation assays is that the binding characteristics of MBL to several micro-organisms might change with the growth phase of the organism (2). We tried to standardize our opsonophagocytosis assay, by harvesting the bacterial and yeast cultures in the log phase. Apart from the growth phase, it has been found that the binding capacity of MBL to different strains of one species may vary considerably (6, 7). Taken together, the opsonophagocytosis assay has its limitations because strains cultured and tested in the lab do not have to be representative for the pathological strains found in patients. Nonetheless, the assay can be a helpful tool to investigate whether an MBL-deficient patient is suffering from an infection because of this lack of MBL, by isolation and culture of the patient’s pathogen itself and testing the opsonophagocytosis of this strain in vitro.

With blocking anti-C1q antibodies and properdin-deficient or factor D-deficient sera we were able to study the lectin pathway-mediated opsonization without interference of the classical or alternative complement pathways. Although ficolins and especially L-ficolin are thought to act on the lectin pathway of complement activation (8), we have not been able to detect L-ficolin-mediated opsonophagocytosis of micro-organisms, even though we used concentrations of purified L-ficolin far above the median serum L-ficolin concentration. So far, L-ficolin-mediated
opsonophagocytosis has only been found for late apoptotic and necrotic host cells, in which L-ficolin enhanced uptake of these altered host cells by macrophages, thereby participating in the maintenance of tissue homeostasis (9). We therefore presume that in our studies, the lectin pathway-mediated opsonophagocytosis of the different micro-organisms was MBL-mediated opsonophagocytosis.

**MBL deficiency as risk factor in immunocompromized individuals: in vivo cohort studies**

To date, it is now generally accepted that MBL deficiency alone is not a risk factor for infection, morbidity or mortality in children or adults. But it may become a risk factor when MBL deficiency occurs concomitantly with: 1) another (humoral) immune deficiency, 2) immune suppression, 3) exposure to unusual infectious challenges. Many of the clinical association studies published in the last 10 years address these patient cohorts. MBL levels and MBL2 genotypes in the patient cohorts have been compared with healthy controls, and intra-cohort associations have been investigated in patients, e.g. prevalence of infections, clinical outcome, morbidity and mortality. Most studies focused on intra-cohort associations, because MBL deficiency is very common in healthy Caucasian controls, hindering the interpretation of clinical implications of increased prevalence of MBL deficiency in the investigated patient groups.

In this thesis we focused on association of MBL deficiency and the clinical outcome in two immuno-suppressed patient groups: (pre-)mature neonates admitted to the neonatal intensive care unit (NICU) and pediatric oncology patients with chemotherapy-induced neutropenia.

Preterm and term neonates are dependent on innate immunity and maternal antibodies and are considered to be prone to develop infections, especially in a NICU setting. MBL deficiency may further increase this risk of infections. We investigated MBL2 genotypes and MBL levels in both umbilical cord blood and neonatal blood. Reduced levels of MBL were found in neonates compared to adult controls, even in neonates expressing a wild-type MBL2 genotype. Reduced MBL levels were associated with younger gestational age, probably because the liver in premature neonates produced insufficient MBL due to immaturity. In neonates with wild-type MBL, or with a heterozygous exon 1 mutation, an increase in MBL levels was seen in the weeks after birth, due to the assumed maturation of the liver. An acute-phase reaction of MBL due to infection can only contribute to this phenomenon in the infected neonates but cannot explain the increase in the uninfected neonates. Although preterm neonates expressed lower levels of MBL, no difference in MBL genotype was found between preterm and term neonates. Thus, MBL2 variant alleles are not a risk factor for prematurity. Although MBL levels found in umbilical cord blood were systematically higher compared to levels in neonatal blood, the MBL levels were highly correlated. This implies that neonates can be investigated for MBL deficiency by the use of umbilical cord blood, thereby sparing the neonates from donating blood.
In a follow-up of this study we associated low levels of MBL at birth with increased risk for early-onset sepsis (within 72 hours after birth), culture-proven sepsis and pneumonia during the first month of life. The association of low MBL levels with younger gestational age, and the association of low MBL levels and increased risk for (nosocomial) infection and sepsis or pneumonia were consistent throughout the several cohorts investigated (10-13). However, the definition of low MBL levels varied among the different cohorts. If the association between low MBL levels in (pre-)term neonates and the risk for developing sepsis in NICU can be confirmed in a multi-centre cohort, with one definition for low or deficient MBL levels (preferentially investigated together with MBL genotype), it will provide a rationale for a placebo-controlled phase-III clinical trial to evaluate the beneficial effects of MBL administration to neonates.

Pediatric oncology patients with chemotherapy-induced neutropenia are also considered to be immune suppressed because of the lack of almost all leukocytes. These patients often suffer from fever during neutropenic episodes (febrile neutropenia) and some patients develop serious infectious complications. MBL deficiency could increase infection susceptibility in neutropenic children and, in turn, administration of purified MBL may therefore reduce the risk of infections during chemotherapy treatment. In a pediatric oncology pilot cohort with chemotherapy-induced neutropenic patients, we did not find an association between MBL deficiency and prevalence or severity of infections. Severe neutropenia (<100 cells/µl) can explain why we did not find an effect of MBL deficiency, because the major effector function of MBL, i.e. enhancement of phagocytosis by opsonization of pathogens, was compromised by the absence of phagocytic cells.

In an extended oncology cohort we determined whether MBL deficiency is a bad prognostic factor in pediatric hematologic cancer and solid tumors for admission to the pediatric intensive care unit (PICU), the time to relapse of malignancy or death. MBL deficiency was associated with longer duration of PICU admissions with febrile neutropenia and with a shorter period to relapse. MBL can bind to late apoptotic and necrotic cells, enhancing their clearance from the body. In MBL-deficient individuals there is a defective clearance of apoptotic cells that may lead to malignancies (14). Therefore, the shorter period to relapse of the malignancy may be apoptosis related. Although we did not find a relation between MBL and risk of infection during febrile neutropenic episodes, we did observe an increase in MBL levels (in wild-type or heterozygous variant MBL patients) during a febrile neutropenic episode, due to the acute-phase response, suggesting lectin pathway activation of complement.

Our results are in agreement with those of others, contradicting the association as previously found between MBL deficiency and increased infection risk during febrile neutropenia (15-17). Differences in age of the patients (adults versus children), the variety in tumor types, chemotherapy regimens, severity of the neutropenia (mild neutropenia <500 cells/µl versus severe neutropenia <100 cells/µl) and the definition of MBL deficiency might all account for these contradicting results. To really establish the role of MBL in chemotherapy-induced neutropenic oncology patients a multi-centre study would be indicated with sufficient statistical power, to
determine the role of MBL levels in patients with similar tumors and/or chemotherapy regimen.

**MBL as a therapeutic agent in pediatric oncology patients**

Substitution of MBL in MBL-deficient individuals with plasma-purified MBL (Statens Serum Institute, MBL-SSI) has proven to be a safe procedure in adults (18) and also in children as we have shown. No serious adverse events occurred and anti-MBL antibodies were not detected 4 weeks to 1 year after the last infusion with MBL-SSI. Anti-MBL antibodies were not expected, because even in sera from donors with homozygous $MBL2$ variant alleles, containing an MBL level below the detection limit of the MBL ELISA, some residual monomeric or dimeric MBL was detected by Western blot analysis. In the MBL substitution study we demonstrated that twice weekly infusions with MBL-SSI in chemotherapy-induced neutropenic oncology patients resulted in MBL trough levels of minimally 1.0 μg/ml. The pharmacokinetic analysis revealed a half-life of 36 hours of the infused MBL. Pharmacokinetics were not related to age after correction of body weight; thus, calculation of the optimal dose of MBL-SSI based on body weight is a reasonable strategy for MBL substitution in MBL-deficient patients.

We investigated the *in vitro* increase of MBL-mediated complement activation and opsonophagocytosis upon MBL substitution *in vivo*, as biological surrogate endpoints for MBL serum reconstitution. Although the desired trough level MBL was thought to be therapeutic at the start of the MBL substitution trial, it appeared that due to the purification process MBL-SSI had suffered a 40% loss of complement-activating capacity, which resulted in suboptimal complement activation and opsonophagocytosis at serum trough levels of 1.0 μg/ml MBL.

Apart from the suboptimal complement activation found at the trough levels, we observed a discrepancy between MBL levels, complement activation and opsonophagocytosis during the first 24 hours after MBL substitution as well. Fifteen minutes after MBL infusion, when circulating MBL levels were at least 3-fold higher than normal circulating serum levels, suboptimal complement C3 activation and opsonophagocytosis of zymosan were detected, although these values were significantly increased compared to the complement activation and opsonophagocytosis before MBL infusion. Further analysis of these samples revealed two causes of this suboptimal result.

The first reason was the suboptimal MASP-2 activation after MBL binding to mannan compared to healthy controls. MASP-2 is a zymogen that becomes activated upon binding to MBL, and this process leads to complement activation to generate C3 convertases. Although we were able to pinpoint the reduced complement activating ability to MASP-2, due to limited patient material we were not able to further study the mechanism responsible for the decrease in MASP-2 activity. There are several explanations possible, however. First, MASP-2 in the circulation is mainly complexed to ficolins and needs to be recruited to subsequently bind MBL, which takes
more than the 15 minutes after MBL infusion, when the earliest blood samples were drawn from the patients.

However, this seems unlikely because after in vitro addition of MBL to MBL-deficient sera we did see optimal reconstitution of complement activation and opsonophagocytosis, without any pre-incubation. Secondly, the MBL-SSI product may be in complex with either inactivated MASP-2 or with inhibitors (C1-inhibitor or α2-macroglobulin), preventing MASP-2 binding to the MBL. But again, the optimal complement activating results obtained after in vitro reconstitution made this hypothesis not very likely either. Finally, the most likely explanation is that the MASP-2 of the patients is altered by the conditions in the patient, either by chemotherapy or MASP-2 synthesis-related perturbations. This alteration could affect the the auto-activation of MASP-2 upon binding to MBL.

The second reason why some patients hardly showed any increase in complement C3 activation or opsonophagocytosis after MBL suppletion was the reduced ability to activate the alternative pathway. Although all patients were analyzed for CH50 and AP50 during screening procedures, apparently some patients became transiently deficient in alternative pathway capacity during chemotherapy. As shown before, the amplification loop of the alternative pathway is important to achieve optimal lectin pathway-mediated opsonization. The reduced or almost absent (in 2 patients) alternative pathway activation capacity seen in the patients participating in the MBL substitution study explains why we did not detect optimal opsonophagocytosis. Unfortunately, due to the limited availability of sera we were not able to investigate which alternative pathway protein was the limiting factor. Properdin is a candidate, because it is released by neutrophils, and the neutropenia in these patients might temporarily reduce the properdin production, resulting in insufficient circulating levels.

Two of the three ongoing phase-IB/-II clinical trials with rhMBL (19) are also being performed in chemotherapy-treated oncology patients, one study in adults, the other in children aged 2-17 years. When the functional efficacy of MBL substitution in these cohorts will be similarly impaired as in our MBL substitution study, not much can be expected of the beneficial effects of MBL substitution in these cohorts.

**Future patient cohorts for clinical studies:**

(Pre-)mature neonates may benefit from MBL substitution, because several studies have shown an increased risk of infection, sepsis and pneumonia in this group of patients. The definition of MBL deficiency should be really strict, because neonates exhibit low MBL levels even with wild-type MBL2 alleles. In the first weeks after birth these levels rise to normal levels for the expressed MBL2 haplotypes. It needs to be further investigated what the exact cut-off level is for insufficient levels of MBL in this cohort and whether lectin pathway (MASP-2) activation
and alternative pathway activation in these patients are normal, to avoid suboptimal efficacy of MBL substitution.

Another patient cohort that will benefit from MBL substitution therapy might be transplant patients with immuno-suppressive therapy, although also in these patient cohorts some contradictory results have been found for the risk of MBL deficiency (20, 21). There are so many contradictory results from patient association studies that it can be doubted whether there will be one specific group of patients that will benefit from MBL as prophylactic therapeutic agent as a whole group. This consideration, in combination with the encountered difficulties in recruiting patients (because the, mainly (very) young, patients experience the twice-weekly MBL infusions as a burden), might implicate that we should not see MBL as prophylactic therapeutic agent, but rather as additional therapy in MBL-deficient patients with recurrent or debilitating infections, for whom regular IVIG or antibiotic treatment alone does not provide the desired effect. These patients might respond favorably to MBL, as was described already for some individual cases (22).

There is a point of concern for MBL substitution that needs to be discussed. Apparently, MBL is not a key component in our immune system, and only under extreme conditions the absence of sufficient levels of MBL will be a risk-factor for disease outcome. The high prevalence of heterozygous MBL2 variant alleles could also imply that there is an advantage to possess intermediate levels of MBL (heterosis). MBL heterozygosity may prevent an overshoot of the immune reaction upon infection, thereby preventing from systemic inflammation and poor disease outcome (23). Thus, before administration of MBL to patients with low MBL levels, it should not only be determined whether MBL substitution is beneficial, but it should also be excluded that it does not lead to over-activation of complement, worsening instead of improving the disease outcome.

Conclusions

Together, the in vitro findings in this thesis describe that in particular yeasts are dependent on lectin pathway-mediated opsonization, more than various strains of bacteria. This implies that in patient studies about the effect of MBL deficiency on infection and infection parameters, a distinction ought to be made between bacterial and yeast infections. For optimal function of MBL in opsonophagocytosis, intact alternative pathway activity is of major importance. The in vivo neonatal MBL association studies showed that newborns have reduced levels of MBL, regardless of MBL2 haplotypes. Low MBL levels in these neonates are correlated with an increased risk of infection, pneumonia and sepsis during NICU uptake. However, in chemotherapy-induced neutropenic pediatric oncology patients, the relation between MBL deficiency and increased infection risk is less clear. Finally, with the MBL substitution study we have shown that MBL infusions are safe for children, and the optimal dose can be calculated from the body weight.
Summary and general discussion

Unfortunately, MBL suppletion did not fully recover complement activation and opsonophagocytosis (in vitro), probably due to the severe neutropenia, causing altered MASP-2 activation and reduced alternative pathway function. This suggests a minimal beneficial effect of MBL suppletion in chemotherapy-induced neutropenic patients.

References


