CHAPTER 1

Introduction

Submitted in part
1. INTRODUCTION

The term immunity is derived from the Latin word *immunitas*, which referred to the exemption from various civic duties and legal prosecution offered to Roman senators during their tenures in office. Since Roman times immunity had been taken to mean freedom from infections, which connoted the ability to resist infection. The immune system is a complex assemblage of cells and molecules, which allow the body to function in a habitat crowded with pathogens.

The immune system can be divided in natural or innate immunity and acquired, adaptive or specific immunity. This general and crude division is based on the ability of the adaptive immune system to increase defense mechanisms in magnitude and specificity following exposure to infectious agents, enabling a more rigorous immune response after a second infection. The innate immunity differs from adaptive immunity as it recognizes a restricted array of structures on a broad range of microorganisms, the so-called pathogen-associated molecular patterns. Repeated contact with pathogens always results in a constant innate immune reaction, which is not amplified.

The ability to vastly counteract a great variety of pathogenic microorganisms is of eminent importance for homeostasis. However, profligate functioning of the immune system is disadvantageous under specific circumstances. Two situations in which excessive immunological response is unwanted are autoimmunity and transplantation. The inability of the immune system to distinguish self-tissue and self-proteins from non-self pathogens (i.e. to maintain of self-tolerance) can result in autoimmune disease causing tissue and organ damage. Traditionally, it is assumed that the recognition of self-determinants in autoimmune diseases is confined to the adaptive immune system, ignoring the role of the innate immune system in auto-immunity. More recently it has been argued that, in order to initiate an (auto)immune response, additional signals generated by the innate immune system (i.e. danger signals) are required (1). Evidence is growing that (auto) antigen recognition by the innate immune system, in a certain context, could lead to autoimmunity, either by priming or promoting aggressive immune responses. In transplantation, disproportionate immune responses can cause both acute and chronic graft rejection as the adaptive immune system may consider the graft hazardous.

The present thesis underscores the current concept of collaboration between the innate and adaptive immune system by showing close interactions between both immune systems. This thesis shows that several constituents of the complement system, a core component of innate immunity, are highly allied with the adaptive immune system. Furthermore the involvement of innate immunity in type 1 diabetes, generally accepted as an adaptive immune system-mediated autoimmune disease is addressed.
In the following paragraphs, a general overview will be given of a specific component of the innate immune system, the complement system. The most recently discovered pathway of the complement system, the lectin pathway and particularly its activating molecule mannose binding lectin (MBL) will be discussed in greater detail. After having outlined the current view on association between MBL and various diseases and disease complications, the role of MBL in transplantation will be addressed. Subsequently, the role of the adaptive immune system will be discussed in pancreatic islet transplantation. Finally, the aim and content of the studies in this thesis will be presented.

2. THE COMPLEMENT SYSTEM

The complement system consists of a variety of functionally linked proteins, including complement factors classified as C1 to C9, which act in conjunction and result in many effects in humoral immunity and inflammation. The five principal biological functions of the complement system are: 1) complement-mediated cytolysis; 2) opsonisation of foreign organisms; 3) activation of inflammation; 4) clearance of self and non-self debris; and 5) amplification of adaptive immunity.

The complement system consists of three pathways, the classical, the alternative and the lectin pathway. All pathways are activated differently, however they converge in a shared terminal pathway and result in the same basic effects. The classical pathway is activated when the first classical pathway component C1 binds to the fragment crystalline (Fc) portion of an immune complex. Activation of the alternative pathway occurs when complement factor C3b binds to various activating surfaces, like microbial cell walls. Finally the lectin pathway, a recently discovered complement activation pathway, is triggered when MBL binds to common carbohydrate structures of a variety of microorganisms (including bacteria, viruses and fungi) (2-6).

Central in the complement system is the complement factor C3. All activated pathways result in generation of a C3 convertase, facilitating the proteolytic cleavage of complement factor C3 into C3a and C3b. Subsequent binding of C3b to C3 convertase enzymes allow the formation of C5 convertase, enabling proteolytic cleavage of the complement factor C5. Upon C5 cleavage, all pathways converge and enter the terminal pathway of complement activation, eventually resulting in the formation of a so-called membrane attack complex (MAC), a lipid-soluble pore structure which causes osmotic lysis of cells (figure 1). Cleavage products C3a and C5a are potent chemotactic products amplifying inflammation. C3b facilitates both opsonisation of foreign organisms and clearance of immune complexes.
As the MBL forms a major topic of the present thesis, the lectin pathway will be discussed in greater detail in the next section.

3. MANNOSE BINDING LECTIN

THE PLURIPOTENT MOLECULE OF THE INNATE IMMUNE SYSTEM

Mannose binding lectin, also referred to as mannann binding lectin or mannan binding protein, is believed to be a central player in the innate immune response. The first case of an association of MBL deficiency and infectious disease dates back to 1968. A small girl, suffering from severe dermatitis, consisting diarrhea and recurrent bacterial infections indifferent to antibiotic and steroid therapy, was reported. Hematological examination revealed a defect in the phagocytosis of yeast particles from *Saccharomyces cerevisiae*, rice starch and *Staphylococcus aureus* in polymorphonuclear leukocytes. This defect was serum-dependent. Infusion of fresh plasma corrected the phagocytic deficiency. As the same phagocytic defect was observed in several direct relatives of the patient, it was concluded that this condition had a genetic origin (7).
In order to fully appreciate the implication of MBL in clinical settings, biological characteristics of MBL will be discussed prior to focusing on the association of MBL with various diseases.

3a. MBL Characteristics

Mannose binding lectin is a C-type serum lectin and is presumed to be produced by the liver. MBL is built up of 96 kDa structural units, which in turn are composed of three identical 32 kDa primary subunits. The subunits consist of an N-terminal cross-linking region, a collagen-like domain and a C-terminal carbohydrate-recognition (CRD) domain (8). Circulating MBL is comprised of higher-order oligomeric structures, which include dimers, trimers, tetramers, pentamers and hexamers of the structural homotrimeric unit. The oligomeric configuration of the structural units allows the MBL molecule to have multiple CRDs facilitating multivalent ligand binding (figure 2). Each CRD of MBL is structurally identical and is able to bind a wide range of oligosaccharides including N-acetylglucosamine, mannose, N-acetylmannosamine and fucose (5). Although the various sugars are bound with different affinities, the cluster-like array of multiple binding sites allows activation of the complement activation to be most effective. MBL is considered to play its major role in innate defense against pathogens, involving recognition of arrays of MBL-binding carbohydrates on microbial surfaces. However, more recent studies have shown that MBL is also involved in the recognition of self-targets, such as apoptotic and necrotic cells.

![Figure 2: Mannose binding lectin (MBL) structural build-up.](image)
In plasma, MBL is associated with MBL-associated serine proteases (MASP) (9). Currently, three MASPs have been identified, MASP-1, MASP-2 and MASP-3 (10-12). Although the function of MASP-1 and MASP-3 remains subject to debate, there is a general consensus of the role of MASP-2, which is responsible for cleavage of C4 and C2, generation of the C3 convertase C4b2a and subsequent complement activation.

Exon 1 of the *mbl-2* gene, which is located at chromosome 10, contains three known single nucleotide polymorphisms (SNPs) at codons 52 (Arg to Cys; allele 'D'), codon 54 (Gly to Asp, allele 'B') and codon 57 (Gly to Glu, allele 'C') (8). All SNPs of exon 1 result in altered collagenous regions and as a consequence, interfere with the formation of high-order oligomers. This impairment of polymerization causes low serum levels of high molecular weight MBL and impaired MBL function. Depending on ethnicity, the allele frequency of variant alleles B, C and D, commonly referred to as O-alleles, may be above 40% (wildtype = A/A) (13). In addition to the three SNPs in exon 1, there are several other polymorphic sites located in the MBL promoter region, including SNPs located at positions -550 (H/L variant), and -221 (X/Y variant), both G to C nucleotide substitutions. Furthermore a polymorphic site is located at position +4 of the 5'-untranslated portion of the *mbl-2* gene (P/Q variant, C→T) (14-16) (figure 3).

The common allele A of exon 1 is associated with the following haplotypes: HYPA,
LYPA, LYQA and LXPA with high, high-intermediate, intermediate and low promoter activity (17). Although there is great variety of MBL levels between the different haplotypes, it has been advocated in order to ease interpretation, to only show the most significant promoter allele in position –221 (X/Y), which is only found in normal A haplotype background (YA or XA) exhibiting high and low promoter activity and serum MBL levels (14). The structural alleles carry the following haplotypes: LYPB, LYQC and HYPD.

3b. MBL and associated diseases

MBL has been studied in a great diversity of diseases. Both decreased and elevated serum levels of MBL and different SNPs of the \textit{mbl2} gene and its promoter have been associated with a variety of diseases, indicating the Jekyll-and-Hyde character of MBL. In order to structure the discussion of this double-edged sword phenomenon, involvement of MBL in different diseases will be discussed according to the aetiology.

\textit{MBL and infectious diseases}

When the adaptive immune response is either immature or compromised, the innate immune system constitutes the principle defense against infection. A logical consequence of impaired MBL function would be an enlarged susceptibility to infectious disease. The phenomenon of an increased incidence of infectious disease in MBL-deficient patients has been shown in pediatric patients and in immune compromised patients. However it also has been shown that adult patients with recurrent infectious disease are more likely to have insufficient serum MBL levels.

\textit{MBL and bacterial infections}

Pediatric patients are still in the developmental stage of the adaptive immune system and rely to a great extent on their innate immune system to counteract infectious pathogens. In support of the theory that MBL has an important protective role in early childhood is a British study amongst 266 pediatric patients (mean age 3.5 years) suffering from meningococcal disease (18). Showing a clinical association between MBL variant alleles and meningococcal disease, the authors of this study suggested that genetic variants of MBL gene might account for a third of all meningococcal disease cases.

Patients undergoing myeloablative bone marrow transplantation or cytotoxic chemotherapy are severely immune-compromised. MBL deficiency has been shown to be associated with severe bacterial infections after chemotherapy and major infections following allogeneic hemopoietic stem cell transplantation (19-21).

In immune-competent Caucasians, it has been suggested that homozygotes for MBL exon 1 codon variants could have an increased risk of invasive pneumococcal
disease (22; 23). Considering post-operative infections a surgical complication, low MBL levels are associated with significantly increased infection rates (24).

In vast contrast to the protective properties of MBL against extracellular bacterial infections is the observation that mycobacterial infections (*Mycobacterium tuberculosis* and *M. leprae*) occur more frequently in patients with increased serum MBL levels. Complement-mediated enhanced phagocytosis as a result of opsonization has been suggested to facilitate these intracellular infections (25).

**MBL and virus infections**

MBL has been studied in relation to various viruses. Persistent hepatitis B virus infection has been reported to be associated with the variant alleles located at codons 52 and 54 of the MBL gene, responsible for low MBL serum levels (26; 27). Furthermore it has been suggested that high MBL serum levels are associated with increased survival rates among Japanese patients with hepatitis B (28). MBL in hepatitis C has been studied to lesser extent and appears to be somewhat contradictory (22; 29-31).

The role of MBL in HIV infection and progression has been a conflict of debate over the last years. The envelope protein gp120 of the HIV-1 virus is highly glycosylated with N-linked carbohydrates, enabling MBL to bind (32; 33). Although it is conceivable that MBL-mediated complement activation could facilitate the immune response directed against HIV infection, reports on the effects of MBL on HIV infection and progression are contradictory.

Although common pediatric virus infection, including RSV and EBV, lack association with MBL (34-36), it has been shown that MBL is able to neutralize the influenza A virus and inhibit the spread of this virus (37; 38).

**MBL and autoimmunity**

It is generally assumed that the recognition of self-determinants is confined to the adaptive immune system, neglecting the role of the innate immune system in autoimmunity. However, evidence is growing that the innate immune system could lead to autoimmunity, either by priming or promoting aggressive immune responses (39; 40). Low MBL serum levels and genetic polymorphisms associated with impaired MBL function have been shown to be associated with different autoimmune diseases including celiac disease and systemic lupus erythematosus (41; 42). A major current pathophysiological concept of autoimmunity is impaired apoptotic cell clearance. MBL has been shown to facilitate the clearance of apoptotic cells (43; 44). A result of cells going into apoptosis is alteration of membrane carbohydrates leading to increased expression of fucose and N-acetyl-glucosamine (45; 46). Redistribution or clustering of glycoproteins has been suggested to enable MBL to bind to these carbohydrates expressed on apoptotic cells, thereby facilitating clearance (47; 48).
When studying the association between MBL and Rheumatoid arthritis, it has been shown that MBL is able to bind to rheumatoid factor (RF) complexes and as a consequence could assist RF clearance by the reticuloendothelial system (49; 50). Although several other studies have not yet been able to reproduce these findings (51), the observations that MBL insufficiency is associated with both elevated IgM RF and increased joint erosions, inflammation and early disease onset are in support of the MBL RF clearance theory (52-56).

**MBL and transplantation**

Tissue damage and impaired organ function as a result of ischemia/reperfusion (I/R) injury still remain enormous predicaments in solid organ transplantation. The hypoxic state to which an organ is subjected during organ harvesting, transport and implantation, result in activation of various immunological events (57-60). The complement system plays an important role in mediating tissue injury after oxidative stress. Activation and deposition of complement on the vascular endothelium following oxidative stress has been shown (61-63) and more interestingly, tissue injury after I/R is significantly reduced by complement inhibition (64; 65). Complement activation via the lectin pathway has been shown following oxidative stress, indicating that inhibition of MBL could be a novel approach in reducing ischemia/reperfusion damage (66;...
67). In support of the involvement of MBL in I/R injury is the fact that MBL-depositions were observed early after transplantation of ischemically injured kidneys (68). Furthermore, it has been suggested that high MBL levels are associated with a more severe form of rejection leading to graft loss in kidney transplantation (69).

**MBL and diabetes**

A major source of mortality and morbidity in diabetes is caused by microvascular complications, as a substantial portion of diabetic patients develop diabetic nephropathy and retinopathy. MBL has been associated with diabetic microvascular complications. Several studies have shown the association between an increased risk of developing renal failure and high MBL producing genotypes in diabetic patients (70-72). The involvement of MBL in the pathogenesis of diabetic nephropathy now appears to be appreciated, however the exact immunological process remains to be studied (figure -4). Controversially, it has been suggested that high MBL serum levels may predict a decreased likelihood of myocardial infarction in diabetic patients (73).

### 4. ADAPTIVE IMMUNITY IN ISLET TRANSPLANTATION

Type 1 (Insulin Dependent) Diabetes Mellitus (T1D) is an autoimmune disease characterized by the specific destruction of beta cells in the pancreas resulting in the loss insulin production. The aetiology of T1D is multifactorial, consisting of genetic predisposition and environmental factors possibly including a variety of viruses and dietary components (74-78). Currently insulin substitution is the most common therapy for patients with T1D. A potential novel therapy for diabetes is transplantation of insulin-producing beta-cells of isolated pancreatic islets. Two major immunological hurdles have to be tackled in order to obtain successful islet transplantation. As the autoimmune response of T1D potentially can destroy the transplanted islets, the recurrence of T-cell autoreactivity against islet determinants, needs to be prevented. In addition, induction of alloimmunity to donor antigens, has to be inhibited (79-81). The attractive and recently successful therapy of transplanting pancreatic islets in T1D is overshadowed by the need for permanent immune suppression. Without the administration of these non-specific and potentially harmful immunosuppressive drugs, graft failure seems inevitable. Islet transplantation is thus limited to diabetic patients already receiving immune suppression for a previous organ transplant, or to patients with severe hypoglycemia unawareness or uncontrollable hyperglycemia. The introduction of a new glucocorticoid-free immunosuppressive regime, the so-called Edmonton protocol, has improved the outcome of islet transplantation considerably (82). This protocol includes sirolimus, tacrolimus and dacluzimab. All
Chapter 1

these immunosuppressive drugs share the same basic quality that they all inhibit T-cell stimulation and proliferation, identifying once again T-cells as key-players in this rejection process (83).

Prediction and prevention of ongoing beta-cell destruction after islet transplantation, resulting in long-term graft survival is of utmost importance. In order to be able to optimise the current islet transplantation, it is essential to study the reaction of T-cells to islets.

5. SCOPE OF THE THESIS

The scope of the current thesis is to obtain insight in immunological aspects of transplantation and diabetes. As previously stated, the present thesis underscores the current concept collaboration between the innate and adaptive immune system by showing close interactions between both immune systems. Mannose binding lectin as a major recognition molecule of the lectin pathway and as a key protein of the immune system was studied in relation to its functional characteristics. Appreciating the Jekyll-and-Hyde character of MBL and the fact that MBL serum levels and functionality are under strict genetic control, MBL was studied under distinct pathological conditions. Chapter 2 describes molecular and biological aspects of mannose binding lectin and the interaction of MBL with the adaptive immune system. The first part focuses on functional characterization of the lectin pathway of complement. The second part discusses the possible compensation of MBL deficiency by antibodies and the classical complement pathway as an example of interaction between adaptive and innate immunity. Furthermore, it describes the functional consequences of genetic MBL polymorphisms for the activation of the lectin pathway in serum of healthy individuals. In the last part of this chapter, lectin pathway complement activation via human IgA is described, presenting a novel linkage between innate and adaptive immunity. Chapter 3 focuses on the involvement of MBL in autoimmunity, by studying juvenile type 1 diabetic patients at disease onset. Prior to starting the study, it was hypothesized that MBL could be associated with type 1 diabetes in three different manners, as shown in figure 4. The genetic predisposition of low MBL serum levels could result in an impaired clearance and inactivation of pathogens responsible for beta cell destruction. Contrarily, high MBL serum levels could contribute to complement activation and inflammation via the recognition of injured tissue, facilitating further beta cell damage and aggravating insulitis progression. Finally, aberrantly glycosylated proteins, as a consequence of the diabetic state, could result in MBL-mediated complement activation resulting in subsequent tissue damage in eyes, kidneys and in various vascular structures (i.e. diabetic complications).
The last theory has been shown in several different studies during the completion of this thesis (70-72; 84). **Chapter 4** addresses the role of the liver in production of serum MBL and to evaluates the effect of MBL variant alleles on the susceptibility to infection after liver transplantation.

Having studied the role of innate immunity in new onset diabetic patients and transplantation, **chapter 5** focuses on the effect of the adaptive immune system on islet transplantation, a novel treatment of type 1 diabetes. The aspects of HLA incompatibility in human pancreatic islet transplantation and the subsequent adaptive allogeneic immune response are discussed. Finally, the results and conclusions of all studies will be summarized and discussed in **chapter 6**.
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