Complement modulation to improve donor organ quality

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Introduction

Renal transplantation

Chronic kidney disease (CKD) is a condition characterized by a gradual loss of kidney function over time. CKD is a progressive disorder that may prelude to the development of end-stage renal disease (ESRD).[1] In The Netherlands, every year about 2.000 patients develop ESRD, which means the kidneys are functioning below 10% of their normal function.[2] Patients with ESRD need timely renal replacement therapy to prevent premature death. In The Netherlands over 6200 patients with ESRD are dependent on dialysis as a form of renal replacement therapy.[3] However, the burden of dialysis is high and patient survival is poor, with a median 5-year survival rate of 45%.[3] Therefore, the optimal treatment for patients with ESRD is renal transplantation. Over the last 25 years, the number of renal transplantations performed in The Netherlands increased from 400 up to almost 1000 renal transplantations annually.[4] However, the number of renal transplantations performed, is limited by the persistent shortage of donor organs. As a consequence the number of patients on the waiting list for renal transplantation is increasing, from 576 patients in 2015 up to 831 patients in 2019.[2]

To narrow the gap between demand and supply it is of importance to optimize and utilize all potential donor kidneys. Nowadays, donor kidneys are retrieved from different types of donors: living donors, donation after circulatory death (DCD) donors and donation after brain death (DBD) donors. The number of kidneys derived from living donors is increasing, especially in The Netherlands, where almost 50% of the kidney grafts are obtained from living donors.[4] This is in contrast to the numbers worldwide, where most kidneys are still retrieved from brain-dead donors.[5,6] However, depending on the donor-type there is a significant variability in the quality of kidneys that are used for transplantation, which results into differences in outcome (Figure 1). Grafts from different types of organ donors are associated with different short- and long-term results post-transplantation. Kidneys derived from brain-dead donors show inferior results compared to kidneys derived from living donors, because of the pathophysiological changes in brain-dead donors.[7–9] Only 45% of the renal grafts derived from brain-dead donors are still functioning 10 years after transplantation, while this is 65% for kidneys derived from living donors.[4]

Next to the standard criteria donors, the donor pool is expanded by the use of extended criteria donors (ECD). Extended criteria donors are deceased donors older than 60 years of age or with an age between 50-60 years with at least two of the following comorbidities: (history of) hypertension, terminal serum creatinine greater than 1.5mg/dl or a cerebrovascular accident as cause of death.[10] These donor characteristics make that ECD-derived kidneys show inferior results compared to kidneys obtained from standard criteria donors.[10,11]

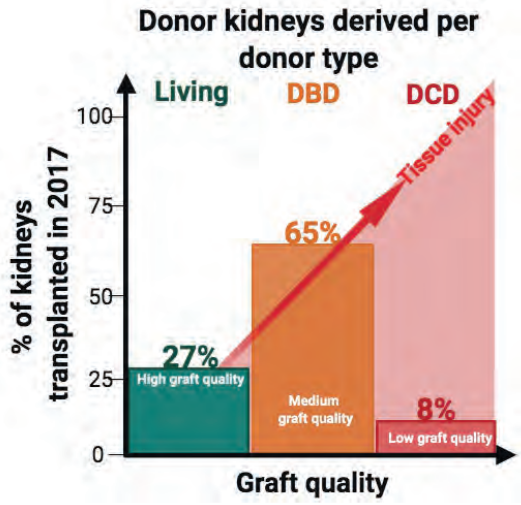

Figure 1. The percentage of kidneys transplanted in 2017 per donor type registered by Eurotransplant.
Data is documented by Eurotransplant and permission for publication was obtained. Data is obtained for all eight countries: Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, The Netherlands and Slovenia. Abbreviations: DBD, donation after brain death; DCD, donation after circulatory death. Created with BioRender.com.

Brain death

Brain death is the irreversible, total loss of brain function, including the brainstem. The three essential criteria to confirm brain death are coma, absence of brainstem reflexes and apnea. Brain-dead donors do not have a nominable warm ischemia time, because the circulation remains intact via mechanical ventilation and cardiovascular support.[12] However, several studies showed that brain death itself should be regarded as a risk factor for deteriorated graft survival.[8,9,13] In contrast to living donors, brain death results in widespread physiological changes such as hemodynamic instability, hormonal changes and a generalized inflammatory response.[14,15]

In all brain-dead patients, increased intracranial pressure (ICP) initially results in an increase in arterial pressure to maintain cerebral perfusion pressure (CPP). If the ICP continues to rise, there is hypoperfusion and eventually ischemia of the brain. This results in sympathetic hyperactivity and is characterized by a catecholamine storm, which is reflected by an immediate increase in mean arterial blood pressure and peripheral vasoconstriction. Peripheral organ injury will be induced by hypoperfusion and ischemia. This sympathetic hyperactivity will eventually stimulate parasympathetic activity, which results in bradycardia. These pathophysiological changes following brain death, characterized by hypertension and bradycardia, is a reflex attempt of the body to maintain CPP, the so-called Cushing's reflex.

In addition, brain death results in a depletion of the hypothalamic-pituitary-axis, which results in the exhaustion of antidiuretic hormone causing excessive loss of fluid, clinically known as diabetes insipidus. Diabetes insipidus during brain death is associated with hypovolemia, hyperosmolarity and hypernatremia.[16] Further, depletion of the hypothalamic-pituitary-axis negatively affects plasma levels of other hormones, such as cortisol, thyroid and adrenocorticotropic hormones. However, the effect of brain death on the endocrinological changes is not uniformly elucidated and seems to depend on the etiology and time course of brain death.[16]

Lastly, brain death elicits a systemic inflammatory response.[17] This is due to the fact that brain death itself results in an initial hyperinflammatory response, resulting in the release of cytokines, priming of polymorphonuclear cells (i.e. neutrophilic granulocytes) and activation of the complement system. The neuroinflammatory response during brain death results in the breakdown of the blood-brain-barrier, which allows cytokines, polymorphonuclear cells and complement activation products to leak into the circulation and trigger a systemic inflammatory response. The systemic inflammation during brain death is characterized by elevated circulating levels of pro-inflammatory mediators such as interleukins IL-1ß, IL-6, IL-8, monocyte chemo attractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α).[18] In addition, regulatory cytokines, such as IL-10 are released during the early phase of brain death. The production of chemokines and adhesion molecules is also stimulated during the inflammatory response in brain death, which is followed by the cellular influx of polymorphonuclear cells in the organs.[17] In this way, the systemic inflammation seen during brain death results in a local intra-organ inflammatory response. Activation of the complement system has been recognized as an important contributor to both the systemic and local inflammatory response seen during brain death.[19–21]

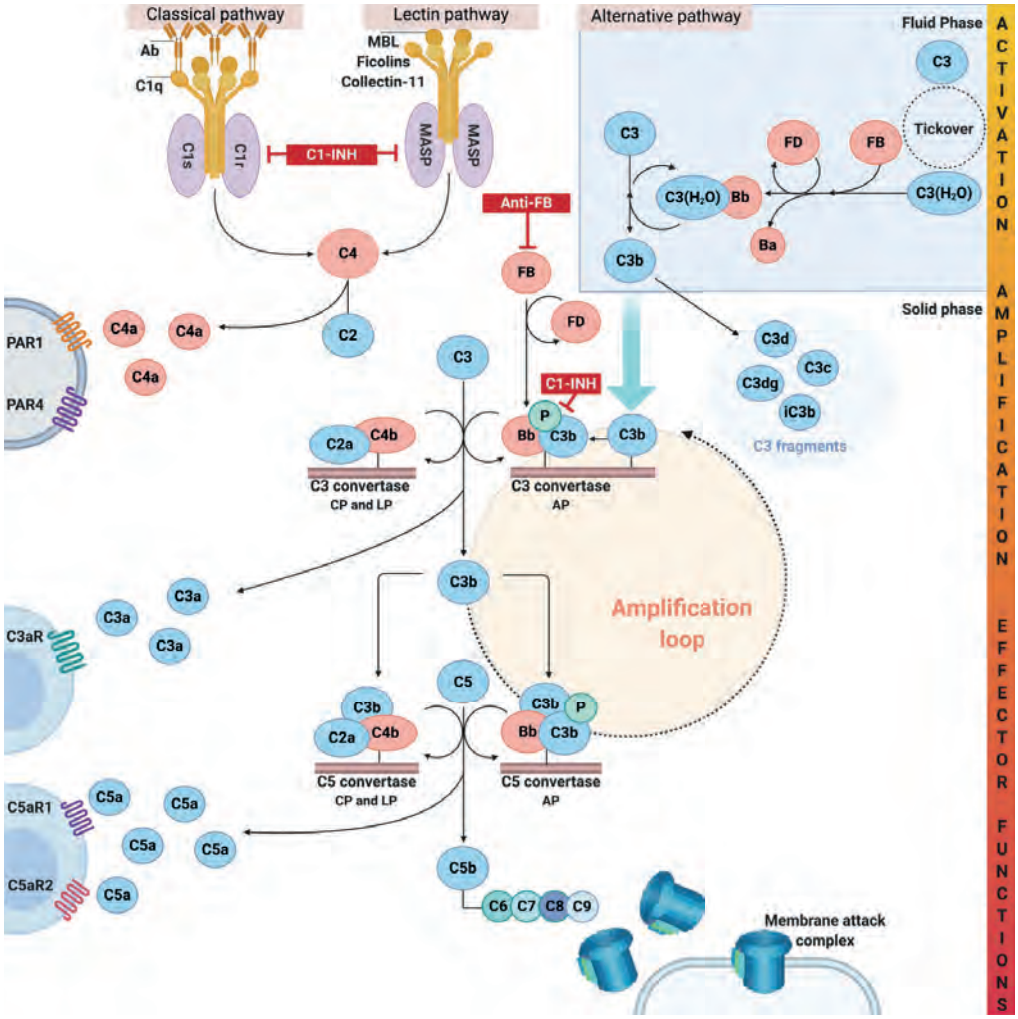

Figure 2. Overview of the complement system.

The complement system is activated by 3 pathways: the classical, lectin and alternative pathway. Activation of each of the three pathways leads to the cleavage of C3 into C3a and C3b and C5 into C5a and C5b respectively. C5b forms together with C6, C7, C8 and C9 a complex named C5b-9, also known as the membrane attack complex. C5b-9 forms a pore in the cell membrane, which will result in cell lysis. In addition, the formation of C5b-9 results in the activation of pro-inflammatory intracellular signaling pathways. Moreover, the anaphylatoxins C3a and C5a are formed. C3a and C5a are effector molecules of the complement system and are strong chemoattractants. C1-inhibitor (C1-INH) and anti-factor B (anti-FB) are complement inhibitors used in chapters 7 and 8 respectively.

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The complement system

The complement system was discovered in the late 19th century by Paul Ehrlich, Jules Bordet and George Nuttal. These bacteriologists identified a heat-labile substance in the serum, which killed bacteria. They called it 'alexine', which means 'to ward off' in Greek. Several years later Ehrlich replaced 'alexine' with 'complement'.[22] The complement system consists of more than 30 membrane-bound and soluble components. Most complement components found in the plasma are synthesized in the liver.[23–25] The complement system is known as an important component of the innate immune system. Activation of the complement system triggers the following immune functions: (i) phagocytosis, by opsonizing antigens; (ii) inflammation, by chemotaxis of polymorphonuclear cells; (iii) cell lysis, by the formation of the membrane attack complex, and (iv) safe clearance of immune complexes, damaged host cells and apoptotic cells. In addition, it forms a bridge between the innate and adaptive immune system, for example by the binding of complement C3b by B cells to promote the secretion of antibodies.[26]

The complement system is activated by three pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP) (Figure 2). The CP is activated when C1q binds to antibodies on a pathogenic surface. C1q binds effectively to IgM, but requires IgG hexamers for similar efficient binding.[27] Further insight has shown antibody-independent activation of the CP via bacterial products, pentraxins (including CRP), apoptotic and necrotic cell components such as annexins, DNA and histones.[28] Binding of C1q to its target results into a conformational change of C1q, which results in the activation of serine proteases C1r and C1s. This enables C1s to cleave its substrate C4 and subsequently C2 is cleaved to form C3 convertase C4b2a. C4b2a cleaves C3 into C3a and C3b, which leads to the formation of the C5 convertase C4b2a3b.[29,30]

LP activation is based on the recognition of pathogens via pattern-recognition molecules (PRM) of the lectin type. Mannose-binding lectin (MBL) was the first PRM of the LP, but currently there are several other known PRMs, including ficolin -1,-2, -3 and collectins.[26] These PRMs recognize carbohydrate structures, which are typically found on bacteria, viruses and fungi. The LP can also bind self-structures as DNA and mitochondria, and participate in the clearance of apoptotic cells. MBL is analogous to C1q of the CP. Binding of MBL to its target results in activation of mannose-associated serine protease (MASP)-1, MASP-2 and MASP-3. MASP-2 is a substitute for C1s and cleaves complement components C4 and C2 to form the C3 convertase C4bC2a. Subsequently C4b2a cleaves C3 into C3a and C3b, which results in the formation of the C5 convertase C4b2a3b.[29,30] MASP-3 is able to convert proenzyme factor D to an active form. So, MASP-3 seems to link the LP and AP of the complement system.[31]

The AP is activated in the fluid phase by the spontaneous hydrolysis of C3 into C3(H_2O), which is also called 'tickover'. The complement system has this 'tickover' to ensure that complement can be quickly activated. After the spontaneous hydrolysis of C3, factor B is recruited and binds to factor D. Then, factor B is cleaved by factor D into Bb and Ba. Binding of Bb to C3(H_2O) forms the AP C3 convertase C3(H_2O)Bb, which will cleave C3 into C3a and C3b. C3b can bind to the surface of invading cells (= opsonization) and bind to factor B and

factor D to form C3 convertase C3bBb.[29,30] In addition, properdin acts as a positive regulator by binding to C3bBb, which stabilizes the C3 convertase. C3bBbP triggers the cleavage of C3 into C3a and C3b, this is the so-called amplification loop. C3bBbP will bind C3b to form the C5 convertase C3bBbC3bP. As a result of the amplification loop, the AP is responsible for the majority of C3a, C3b, C5a and C5b-9 formation, independent from the pathway of activation.[32]

All three pathways merge at the point of C3. Activation of C3 by the cleavage into C3a and C3b marks the start of terminal pathway activation. Subsequently C5 is cleaved into C5a and C5b. C5b associates with C6, C7, C8 and multiple C9 components, to form the membrane attack complex (MAC) C5b-9. C5b-9 forms a pore in the cell membrane, which results in cell lysis. In addition, C5b-9 activates pro-inflammatory intracellular signaling pathways. In case the terminal pathway is activated in the fluid phase, soluble C5b-9 (sC5b-9) is formed, which can be measured in the plasma.

In addition to the formation of C5b-9, the anaphylatoxins C3a and C5a are formed during complement terminal pathway activation. C3a is an anaphylatoxin that binds to its receptor C3aR, resulting in both pro-inflammatory and anti-inflammatory signaling.[33] C5a is known to be the most potent anaphylatoxin of the complement system. There are two known C5a receptors: C5aR1 and C5aR2, which are both expressed on immune cells as well as renal tubular cells.[34] C5aR1 is a G protein-coupled membrane-bound receptor. C5aR2 is not coupled to G proteins and therefore the function of C5aR2 is debated. C5aR2 is described to modulate C5aR1 signaling, which can induce anti- or pro-inflammatory responses. In case C5aR2 controls the availability of C5a, it might limit the C5a-C5aR1-axis and act as an anti-inflammatory scavenger. However, C5aR2 might be able to induce G-protein independent activation of immune cells, which can result in a pro-inflammatory response.[35]

Complement in brain death

So, the complement system has multiple effector functions, which could potentially harm healthy tissue and cells. Therefore, it is essential that complement activation is strictly regulated. To do so, there are several circulating plasma and membrane-bound complement regulators, which regulate the location and activity of various complement factors.[36] During brain death, this fine balance between complement activation and regulation is disturbed, which results in organ injury.

During brain death the complement system is activated, which results in increased circulating levels of complement components Bb, C4a, C5a and C5b-9.[20,37] These increased systemic C5b-9 levels in brain-dead donors are associated with inferior renal allograft function and a higher incidence of acute rejection in the recipient.[20] In addition, in the setting of lung transplantation it has been demonstrated that increased circulating complement levels are associated with an increased risk for primary graft dysfunction after transplantation.[38] So, complement is already activated in brain-dead donors, and systemic complement activation may trigger a local inflammatory response, which results in priming of the donor organ prior to transplantation.

More than 20 years ago, complement deposition as a result of brain death was first described. Complement C3 deposition was detectable 1-hour post-transplantation in kidneys derived from a brain-dead donor, while no C3 deposition was seen in kidneys derived from a living donor.[13] In addition, brain death itself elicits local synthesis of complement by the renal graft, as demonstrated in both murine and human studies.[19,39] Local complement deposition in renal grafts is associated with inferior graft function in the recipient.[19] Studies in other organs, such as heart and lungs, confirmed the local deposition of complement during brain death.[40–42] Altogether, based on the current knowledge, brain death leads to systemic and local complement activation, which both seem to be associated with an inferior outcome after transplantation.

Interestingly, less is known about how complement is activated in brain death. However, there are several hypotheses on how the complement system could be systemically activated during brain death. First, it is hypothesized that brain death initiates a sterile immune response through the release of endogenous damage-associated molecular patterns (DAMPs). These danger signals are released by cells under conditions of cellular stress or tissue injury. In brain-dead donors, these endogenous DAMPs are either actively secreted via stressed immune cells or passively released from dying brain cells or damaged extracellular matrix. This can contribute to CP activation via pre-existing naturally occurring IgM antibodies. Second, it is known that sterile inflammation can also cause further tissue destruction by the release of excessive amounts of DAMPs via necrotic cells. As a result, an extension of the local inflammation to the systemic circulation occurs, in a similar manner as is seen during a microbial invasion. C1q and properdin will bind to these necrotic cells, which triggers the activation of the complement system via the CP and AP respectively. In addition, brain death causes an increase in intestinal permeability, which results in the release of pathogen-

associated molecular pattern molecules (PAMPs), such as lipopolysaccharide (LPS). In this way, the complement system is activated via the AP.

However, profound knowledge about how complement is activated in brain death is lacking, especially with regard to the contribution of the complement activation pathways in different donor organs. It is important to elucidate how complement is activated and mediates brain death-induced organ injury to support the development of potential therapies.

Rationale

The aim of this thesis is to elucidate the contribution of the different complement pathways in brain death-induced organ injury, especially in the setting of renal transplantation. The last decade, complement activation has shown to play an essential role in renal (allo)graft injury. However, less is known about the pathway(s) responsible for complement-mediated renal injury during brain death. Profound knowledge of the underlying pathological processes is essential to enable optimal target and drug selection in order to improve renal graft quality prior to transplantation. As the field of complement therapeutics emerges, with currently more than 20 complement therapeutics under development, the goal of using complement therapeutics to improve renal graft quality prior to transplantation is becoming more achievable. Therefore, the research described in this thesis aims to identify the optimal targets to improve the quality of renal grafts derived from brain-dead donors prior to transplantation.

The complement system is activated during all stages of renal transplantation: in the brain-dead donor, during preservation, at time of ischemia and reperfusion, and in the recipient after transplantation. **Chapter 2** provides an overview of the current knowledge about the role of the complement system during all these phases of renal transplantation. In addition, it focusses on the current knowledge gaps and highlights the potential of complement modulation during different phases of renal transplantation. In **chapter 3** the contribution of the complement pathways in brain death-induced renal injury and inflammation was dissected by using a mouse model of brain death. To determine the effect of brain death-induced complement activation on renal injury, C3-deficient mice were subjected to brain death. Second, the role of the C5a-C5aR-axis was investigated by subjecting C5aR1- and C5aR2-deficient mice to brain death. Lastly, the role of the different complement activation pathways was dissected by subjecting C4-deficient (central component of CP and LP activation) and properdin-deficient mice (essential for AP activation) to brain death.

Since complement activation already commences in the brain-dead donor, not only kidneys, but also other donor organs, such as heart, lung, liver and intestines, could be damaged as a result of complement activation. The exact role of complement activation and differences in the contribution of the complement activation pathways in different donor organs have not been elucidated. The physiological differences between organs might lead to various routes of immunological activation and therefore require different therapeutic approaches. Therefore, in **chapter 4** the role of the complement activation pathways in brain death-induced lung injury and inflammation was dissected by subjecting wildtype, C3-, C4- and properdin-deficient mice to brain death.

The next phase of transplantation is preservation of the donor organs. The field of preservation made huge progress over the last decade by the development and implementation of a new preservation method: machine perfusion. Machine perfusion is a preservation technique in which the donor organ is perfused with a (blood-based) oxygenated solution at a hypothermic or normothermic temperature. Since the twenty-first century, hypothermic machine perfusion (HMP) has progressed to a clinical phase, where it now has obtained its place in standard care. Normothermic machine perfusion (NMP) is still in the early

preclinical phase, but might be a promising preservation strategy because it reconstitutes a near-physiological environment. So far, less is known about the immunological and inflammatory changes in the donor kidney following reperfusion in NMP. In **chapter 5** the immunological and inflammatory response of the donor kidney during NMP was investigated. For this purpose, NMP was performed with porcine and human discarded donor kidneys for 4 and 6 hours respectively.

In parallel to the developments in the field of transplantation, an overwhelming number of complement therapeutics has been developed over the past 10 years. **Chapter 6** provides an overview of the current knowledge about complement activation and complement therapeutics in the multi-organ donor. In addition, new potential complement targets are highlighted and existing challenges are described. Next, in **chapter 7** and **chapter 8,** two promising complement inhibitors were examined as donor treatment in a rat model of brain death. In **chapter 7**, complement C1-inhibitor (C1-INH) was administered 30 minutes after the confirmation of brain death and its effect on renal injury and inflammation was evaluated after four hours of brain death. C1-INH is a central regulator of the complement system, as it inactivates C1r and C1s of the CP and MASP-1 and MASP-2 of the LP. Recently published studies demonstrated that C1-INH can act as a down regulator of AP activation as well.[43,44] However, C1-INH also inactivates serine proteases of the coagulation system and is therefore not a specific complement inhibitor. In **chapter 8**, the use of a monoclonal antibody against factor B (anti-FB) in order to reduce brain death-induced renal injury was examined in a rat model of brain death. Factor B is an essential component of the AP, because this pathway plays a major role in amplifying the complement response. Therefore, therapeutic strategies that target the AP could offer a robust strategy to inhibit complement activation in brain-dead donors. To conclude this thesis, in **chapter 9** the obtained results are summarized and discussed, and it furthermore describes current challenges and considerations for future research.

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