Complement activation and effector pathways in ischaemia reperfusion injury and at the interface with adaptive immunity

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Complement activation and effector pathways in ischaemia reperfusion injury and at the interface with adaptive immunity

By

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A thesis submitted to the School of Medicine at King's College London for the degree of Doctor of Philosophy

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May 2013

Declaration

The work presented in this thesis is my own and except where acknowledged in the text, all experiments were performed by myself.

Abstract

Ischaemia reperfusion injury (IRI) is an important source of morbidity and mortality and contributes to renal transplant failure. Renal IRI is known to be mediated by complement, which causes direct injury to the transplant and stimulates the adaptive immune response against transplant antigens. However uncertainty exists as to the exact trigger mechanisms of complement activation in this situation, and to the relative importance of certain effector molecules generated by complement activation.

Based on the results of previous studies, I hypothesized that there would be greater involvement of the lectin pathway than previously recognized, as a trigger mechanism that could both initiate complement activation and lead to amplification via the alternative pathway in renal IRI. I also predicted that, whereas C5a has been shown to play a part in the genesis of IRI, C3a could be shown to have an important effect on innate cells that form a bridge with adaptive immunity. The aim of the work described in this thesis was therefore to investigate: (a) the role of the lectin pathway in IRI through the enzyme MASP-2; and (b) the role of C3a linking up the innate and adaptive immune responses following transplantation.

I first investigated renal IRI in mice with deficiencies of the classical (C4), alternative (FB) and lectin (MASP-2) pathways of complement activation in order to explore the notion that the injury was mediated by the lectin pathway. Absence of C4 and FB did not protect the mice in a transplant model of IRI; but absence of MASP-2 was markedly protective suggesting that the lectin pathway - or rather a variant of it that did not involve C4 - has a significant role in the mouse kidney

transplant IRI. As a result, I also investigated the therapeutic effect of anti-MASP-2 antibody in the mouse kidney isograft model.

Absence of C3a-receptor in a native kidney model of IRI did not convey a substantial protective effect, suggesting no major effect of C3a in this injury. However, exploring a possible effect of C3a on the adaptive immune response, I found that stimulation of monocytes with C3a in conjunction with LPS resulted in significant IL-1β production which in turn induced Th-17 cells. I identified that the increase in monocyte IL-1β production is ATP and caspase-1 dependent and that the change in ATP is modulated by Pannexin-1 channel.

My findings identify MASP-2 as an early step in the pathogenesis of complement-mediated mouse kidney IRI and support its identity as a therapeutic target. Furthermore, as both monocytes and Th-17 cells have been previously identified as major contributors to acute kidney rejection after transplantation, my results could explain, at least in part, how complement activation induced by tissue stress during the transplant procedure has an impact on the alloimmune response.

Acknowledgement

I would like to express my sincere gratitude to my supervisors Professor Steven Sacks, Dr Claudia Kemper and Professor Wilhelm Schwaeble for their invaluable academic guidance and support throughout this project. I would also like to thank Dr Wuding Zhou for her supervision and advice in the first year of my project and for helping me set up the native mouse ischaemia reperfusion model and learn various laboratory skills with assistance of some of her group members in particular Dr Qi Peng and Dr Ke Li. My special thanks goes to Dr Conrad Farrar who patiently taught me the technique of inducing native kidney ischaemia reperfusion injury as well as many other laboratory skills, performed most of the mouse kidney transplant experiments in this project and helped with scoring of histology samples.

Several other members of the lab have contributed to the work presented here that I would like to acknowledge and express my great appreciation to: Dr Qijun Li for performing the kidney transplants for assessing the therapeutic effect of anti-MASP-2, Dr Gaelle Le Friec for her constant presence and great patience in teaching me laboratory skills and helping me with experiments, Dr Richard Smith for sharing some of his profound knowledge of biochemistry and complement and providing guidance and help in some of the experiments, Dr Hidekazu Yamamoto for his help in staining human kidney transplants and confocal microscopy, Dr John Harris from the NIKON Imaging Centre at KCL for his help in the generation of the confocal microscopy images and Dr Esperanza Perucha for providing the Th17 skewing protocol. I am also grateful to Dr Sarah de Freitas for her support throughout the project.

A number of people outside of the lab have also made contribution to this project to whom I would like to express my gratitude: Professor Terrence Cook for providing the rejecting human kidney transplant tissue, Professor Jörg Köhl for his advice on the monocye and anaphylatoxin experiments, Omeros Corporation (Seatle) for providing anti-MASP-2 antibody and partial funding for the experiments related to the use of anti-MASP-2 in mouse kidney transplantation and from Professor Schwaeble's lab in Leicester, Dr Youssif M Ali for providing the mouse anti-MASP-2 antibody and Elvina Chrysanthou and Dr Hany Kenawy for helping with MASP-2 staining in the mouse kidney and liver tissue.

Most importantly I would like to acknowledge and thank Kidney Research UK and MRC Centre for Transplantation at King's College London for providing the funding to carry out the work for this thesis.

Finally I would like to thank my husband for his patience and continuous support during my work on this project and writing up this thesis, my parents for believing in me and my friends and colleagues for their understanding and encouragement.

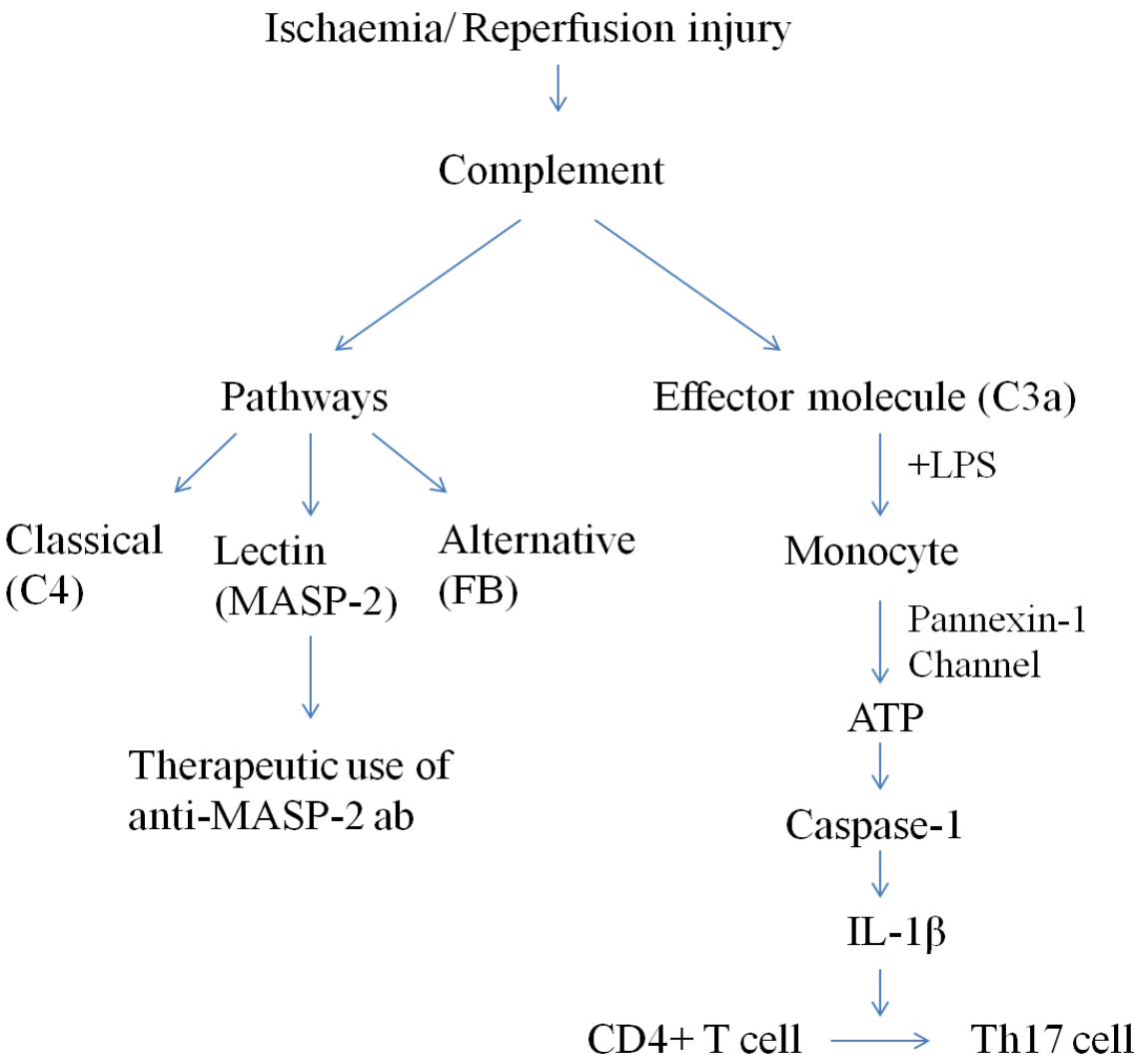

Figure 1. Outline of the research project. This figure shows the outline of the research project. I investigated the role of C3a (native) and classical, lectin and alternative pathways of complement (transplant) in kidney IRI. I then assessed the influence of C3a on the adaptive immune response in kidney transplant rejection and the mechanism of its action.

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List of abbreviations

AP	Alternative pathway
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BBS	Barbital buffered saline
BSA	Bovine serum albumin
BUN	Blood Urea Nitrogen
C3aR	C3a receptor
C5aR	C5a receptor
C4BP	C4b-binding protein
CRD	Carbohydrate recognition domain
CRP	C- reactive protein
CD	Cluster of differentiation
CP	Classical pathway
CVF	Cobra Venom Factor
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
DGF	Delayed Graft Function
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxyribonucleoside triphosphate mixture
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H & E	Haematoxylin and Eosin stain
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase

HMGB1	High-mobility group box 1
HSP	Henoch Schönlein purpura
HSPs	Heat shock proteins
ICAM-1	Intercellular cell adhesion molecule–1
IP	Intraperitoneal
IV	Intravenous
IFN	Interferon
IL	Interleukin
IRI	Ischaemia reperfusion injury
LP	Lectin pathway
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MHC	Major histocompatibility complex
MAp19	MBL-associated protein of 19 kD
MASP	Mannan associated serine protease
MBL	Mannan binding lectin
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic acid-Schiff's base stain
PMNs	Polymorphonuclear leukocytes
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute medium
RT-qPCR	Real-time quantitative polymerase chain reaction
SDS-PAGE	SDS poly-acrylamide gel electrophoresis
SEM	Standard error of the mean
TBE	Tris-Borate-EDTA
TBS	Tris buffered saline
TGF-β	Transforming growth factor β

Th	T helper
TLRs	Toll like receptors
TNF	Tumour necrosis factor
VCAM-1	Vascular cell adhesion molecule–1
WT	Wild type

1 Introduction

1.1 Ischaemia Reperfusion injury

Ischaemia is defined as restriction of blood supply to an organ which results in deprivation of oxygen and nutrients leading to tissue damage. It causes significant morbidity and mortality in several conditions such as myocardial infarction, stroke, intestinal and limb ischaemia and cardiopulmonary bypass (Zhou et al., 2000). Restoration of blood flow to the ischemic tissue is known as reperfusion which despite restoring tissue oxygenation leads to adverse reactions and inflammation (Yellon and Hausenloy, 2007). Ischaemia reperfusion injury (IRI) refers to the tissue damage that occurs following re-establishment of blood flow after an ischaemic episode (de Vries et al., 2009).

IRI is an important cause of kidney damage in hypovolaemic acute renal failure (Mejia-Vilet et al., 2007) as well as playing a significant role in delayed graft function (DGF) following kidney transplantation (Gueler et al., 2004). It has also been shown to increase the acute rejection episodes and reduce long term graft survival. In an analysis of 27,096 first cadaveric donor renal transplants, Shoskes and colleagues (Shoskes and Cecka, 1998) reported an increase in the incidence of acute rejection in patients with DGF before discharge (25% in comparison to 8% in patients without DGF) and a reduction in 1-year graft survival from 91 to 75% independent of early rejection episodes. Despite many studies investigating the mechanism of IRI, there is no specific therapy for this condition that is in clinical use.

Preventing the occurrence of IRI in kidney transplantation can reduce the injury to the donor kidney, reduce rejection episodes and prolong kidney graft survival (Bryan et al., 2001, Ojo et al., 1997).

1.2 Pathophysiology of IRI

Pathophysiology of IRI is complex and involves a cascade of events including interaction between endothelial cells, interstitium, immune cells and several biochemical molecules (Linfert et al., 2009) leading to what is now established as an inflammatory response.

Activation of the endothelium also follows the acute ischaemic event leading to an increase in endothelial permeability and the expression of various adhesion molecules (Halloran et al., 1997). These adhesion molecules such as intercellular cell adhesion molecule–1 (ICAM-1), vascular cell adhesion molecule–1 (VCAM-1) and P-selectin initiate the recruitment and infiltration of inflammatory cells to the injured tissue (Turer and Hill, 2010). Injury to endothelial cells increases their thrombogenic capacity which results in adhesion of platelet and inflammatory cells upon reperfusion. The inflammatory cells release cytokines as well as reactive oxygen species (ROS) which potentiates the inflammatory reaction (Gueler et al., 2004).

Cellular apoptosis and necrosis occurs due to ischaemia, leading to the exposure or release of Damage Associated Molecular Patterns (DAMPs) including chromatin-associated protein high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), and purine metabolites such as ATP (Iyer et al., 2009). DAMPs can activate pattern recognition receptors such as the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD)-like receptors which can lead to induction and activation of transcription factors like nuclear factor-κB (NF-κB) and mitogen-

activated protein kinase (MAPK) and consequently up-regulate genes that are involved in inflammatory responses (Chen and Nunez, 2010). Hypoxic and anoxic cell injury due to ischaemia also causes dramatic changes in epithelial and endothelial cell morphology and protein/receptor surface expression profile. Following reperfusion, elements of the innate immune system within the blood recognize the ischaemia-induced changes on exposed cells resulting in increased tissue injury (Jang and Rabb, 2009).

The most studied effector cells in IRI are neutrophils followed by macrophages which in combination with other elements of innate immunity such as the complement system have the most significant impact in inducing tissue damage. It has been shown more recently that the adaptive immune system including T and B cells also play an important role in IRI (Linfert et al., 2009, Satpute et al., 2009, Renner et al., 2010).

1.3 The complement system

The complement system, which is an important mediator of the innate immune defense, has diverse roles including elimination of pathogenic bacteria, interaction between innate and adaptive immunity and clearance of immune complexes and cellular debris following an inflammatory injury (Diepenhorst et al., 2009).

Complement was initially described in the 1890s as a heat-labile compound in serum that 'complemented' the heat-stable antibodies in the killing of bacteria (Walport, 2001). Since then, 35 to 40 cell bound or soluble proteins and glycoproteins with activating or regulatory functions have been described in the complement system (Carroll and Sim, 2011). The soluble complement components are usually in the form of pro-enzymes which become activated following a proteolytic cleavage. This

process initiates a cascade of enzymatic activation leading to production of anaphylatoxins and other biologically active material which can damage cells. Several regulatory proteins are responsible for protection of host cells from this damage.

The complement system is activated via three distinguishable pathways known as the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP).

The CP is antibody-dependent and is activated following binding of C1q within the C1 complex to antigen-antibody complexes. The AP is activated when activated C3 (C3b) binds to pathogens or other potential activating surfaces in the presence of factor B and factor D. Activated C3 is formed spontaneously in plasma through cleavage of C3 in a process which is called C3 tick over (Lachmann and Halbwachs, 1975). In the LP which has been characterized more recently, pattern recognition molecules such as mannan binding lectin (MBL), ficolins and CL-11 (collectin-11) bind to specific carbohydrate residues containing mannose on bacteria, viruses and eukaryotic pathogens or altered self cells, such as apoptotic, necrotic or malignant cells after which the enzymatic activity of MBL-associated serine proteases (MASPs) triggers the activation of the pathway (Ali et al., 2012, Fujita, 2002, Collard et al., 2000b). Various coagulation factors and serine proteases belonging to the coagulation system such as FXa, FXIa, plasmin and thrombin have also been shown to be able to cleave C3 and/or C5 molecule and initiate the complement pathway activation (Amara et al., 2008). All complement pathways merge at the level of the complement component C3. With formation of C3 convertase and C5 convertase, the terminal pathway components are produced leading to the formation of C5b-9 which is known as the membrane attack complex (MAC) that is responsible for the final cell or pathogen damage by creating a transmembrane pore (Peitsch and

Tschopp, 1991). In the process of complement cascade activation, several by-products are made from the complement molecules such as the anaphylatoxins C3a and C5a (Hugli, 1984). These have been shown to have both inflammatory and immunomodulatory roles. Figure 2 summarizes the key element of the complement activation pathways.

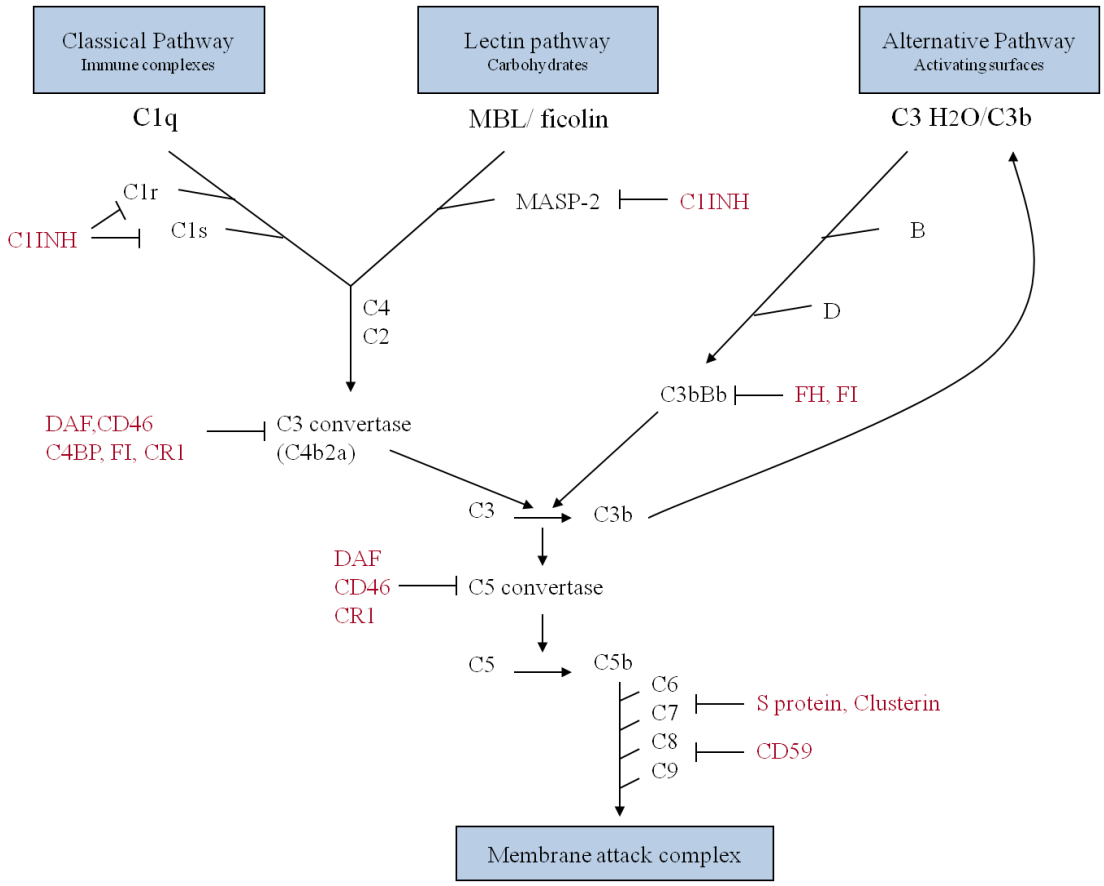


Figure 2. Schematic of the complement activation pathways. This schematic shows the molecules involved and the sequence of events in classical, lectin and alternative pathway of complement activation. The red colour indicates complement regulatory molecules. C4BP, C4b binding protein; CR1, complement receptor type 1; DAF, decay accelerating factor, C1INH, C1-inhibitor; FH, factor H; FI, factor I

1.3.1 The Classical pathway

The classical pathway of complement becomes activated when an antibody (IgM and complement fixing isotypes of IgG) is produced against antigens such as bacteria or

viruses and forms an antigen-antibody complex. Several other factors have been shown to initiate the CP activation such as certain bacteria, viruses, endotoxins, bacterial lipopolysaccharide (LPS), C-reactive protein and apoptotic cells (Navratil et al., 2001, Alberti et al., 1996, Mihlan et al., 2011).

The initiating factor in the classical pathway activation is the 790 kDa C1 complex that is made up of one C1q molecule and two Ca^{2+} dependent protease molecules C1r and C1s which are formed as a tetramer C1s-C1r-C1r-C1s (Arlaud et al., 2001). C1q comprises 460 kDa of the C1 molecule and is its recognition component. It has 18 polypeptide chains coded by three different genes and is organized into a structure containing globular domains. Simultaneous binding of these globular heads to triggering ligands results in conformational changes in the C1q molecule leading to auto-activation of C1r. This activates the C1s protease which then cleaves the next two complement components, C4 and C2. Cleavage of C4 produces a small C4a fragment which is released in the fluid phase and a larger C4b fragment that is able to covalently bind to a substrate such as the surface of pathogens. After binding to a target, C4b can function as an opsonin or form a complex with C2. After C2 binds to C4b, it gets cleaved by C1s producing a small fragment C2b and a large fragment C2a that remains attached to C4b and forms the C3-convertase C4b2a.

The C3-convertase then cleaves the C3 molecules to form a small C3a molecule and C3b. C3a is an anaphylatoxin which induces a local inflammatory response and attracts various immune cells to the site of inflammation. Removal of C3a results in a conformational change in the C3b portion, allowing it to covalently bind to the surface of pathogens or damaged cells and facilitate their phagocytosis and disposal by host cells that have C3b receptors. C3b also binds to the formed C3-convertase to make the complex C4b2a3b which acts as C5 convertase. This convertase then

cleaves the C5 molecule to C5a, a potent anaphylatoxin, and C5b which can initiate the assembly of the membrane attack complex (MAC).

1.3.2 The Alternative Pathway

The alternative pathway of complement, in contrast to the classic and lectin pathways, does not require a specific recognition complex for its activation. The automatic and continuous low level hydrolysis of the thioester bond in C3 (known as 'tickover') that forms C3(H2O) is the initiating step in its activation (Thurman and Holers, 2006, Lachmann and Halbwachs, 1975). Molecular conformation of C3(H2O) is similar to C3b which can bind to factor B, making it susceptible to cleavage by serum protease factor D, producing Ba and Bb. Bb remains attached to C3(H2O), forming the fluid phase C3 convertase which can cleave C3 molecules to C3a and C3b. The C3b associates with factor B and produces the alternative pathway C3 convertase, C3bBb, creating a positive feedback loop. The convertase is stabilized by another complement component, properdin, which is a positive complement regulatory factor (Hourcade, 2006). This positive feedback loop can also act on the C3b produced in the CP or LP and amplify their activation.

Most of C3b is inactivated through hydrolysis; however, in the presence of an activating target such as a pathogen, C3b covalently binds to its surface and acts as an opsonin. C3b can also bind to the C3 convertase and form a complex known as C3b2Bb which acts as the alternative pathway C5 convertase, cleaving C5 molecules, leading to initiation of the terminal pathway. C3b also binds to host cells, in which case several soluble and membrane bound complement regulatory proteins come into action to prevent complement activation.

1.3.3 The Lectin Pathway

The lectin pathway (also known as mannan binding lectin pathway) is the most recent complement pathway to be described (Ikeda et al., 1987), although it is thought to have existed prior to both classic and alternative pathways and represents an ancient mechanism of host defense.

The LP becomes activated when the pattern recognition molecules such as mannan binding lectin (MBL) or ficolins recognize certain carbohydrates or N-acetyl groups on the surface of pathogens. Binding of MBL or ficolins to the microbial surface causes activation of MBL-associated proteases known as MASP-1, MASP-2 and MASP-3. MASP-2 acts similar to C1s of the CP and is able to cleave C4 and C2 molecules and form the C3-convertase C4b2a which then cleaves the C3 molecule, whereas MASP-1 can cleave C3 directly, leading to activation of the AP (Fujita et al., 2004b).

The CP and LP have several characteristics in common in that they both have a pattern recognition molecule (MBL and ficolins in the LP and C1q in the CP) which upon binding to a target activates a protease leading to the formation of the C3 convertase C4b2a (Gal et al., 2007). The serum proteases of the 2 pathways (C1r and C1s of the CP and MASP enzymes in the LP) are also structurally related and both bind the recognition molecules in a Ca^{2+} dependent interaction (Phillips et al., 2009).

1.3.3.1 Collectins and ficolins

The evolutionarily conserved immune system has maintained an effective first line defense against invading pathogens. Pattern recognition proteins act in this first line by recognizing and binding the pathogen-associated molecular patterns (PAMPs) on the surface of microorganisms and initiating the innate immune response. Collectins

and ficolins are two important groups of pattern recognition molecules present in plasma and mucosal surfaces that are able to recognize PAMPs (Holmskov et al., 2003). They both have a collagen-like sequence at their N-terminal which allows the assembly of several polypeptides and formation of oligomeric structures. The C-terminal of collectins and ficolins which is the carbohydrate recognition domain (CRD) consists of C-lectin in collectin molecules and fibrinogen in ficolins (Lu et al., 2002). Binding of the CRD segment of collectins and ficolins to carbohydrates on pathogens and their collagen-like region to ligands on immune cells, stimulates innate immune responses such as phagocytosis and/or complement activation and leads to clearance of the microorganism (Holmskov et al., 1994).

The name collectin was proposed for a family of pattern recognition receptors that have structural and functional similarities to C1q of the classical pathway (Malhotra et al., 1992). MBL and surfactant proteins A and D (SP-A and SP-D respectively) are members of the collectin family whose functions have been well studied. MBL which is produced in the liver is a serum protein that together with MBL-associated serine proteases is able to activate the lectin pathway of complement (Kawasaki et al., 1983, Sato et al., 1994). SP-A and SP-D proteins are mainly synthesized by type II alveolar cells and were initially isolated from the lung surfactant (White et al., 1985, Persson et al., 1988). They play an important role in host defense by modulating leukocyte function as well as enhancing the killing and removal of pathogens via opsonisation (Crouch et al., 2000). Several other human collectins have been identified including collectin liver 1 (CL-L1) (Ohtani et al., 1999), membrane bound collectin placenta 1 (CL-P1) (Ohtani et al., 2001) and more recently collectin 11 (Keshi et al., 2006).

I have concentrated on MBL and ficolins which are most relevant to my project with a brief touch on collectin 11 which has exciting potential for future studies related to kidney disease.

1.3.3.2 Molecular characteristics of MBL, ficolins and MASPs

MBL and ficolins are pattern/danger recognition molecules that can bind to specific highly evolutionarily conserved patterns on the surface of certain pathogens and also altered host cells (Fujita et al., 2004b). They belong to the large family of lectins which are characterized by the presence of a collagen-like domain and a carbohydrate-binding domain (Figure 2) (Fujita, 2002). They are both oligomers of structural subunits that contain three identical polypeptides (32 kDa in MBL and 35 kDa in ficolins). The polypeptide chain consists of a cysteine-rich N-terminal, a neck region and a collagen-like domain. The ligand binding sites in these molecules are different and consist of a C-type carbohydrate recognition domain in MBL and a fibrinogen like domain in ficolin (Matsushita et al., 2000).

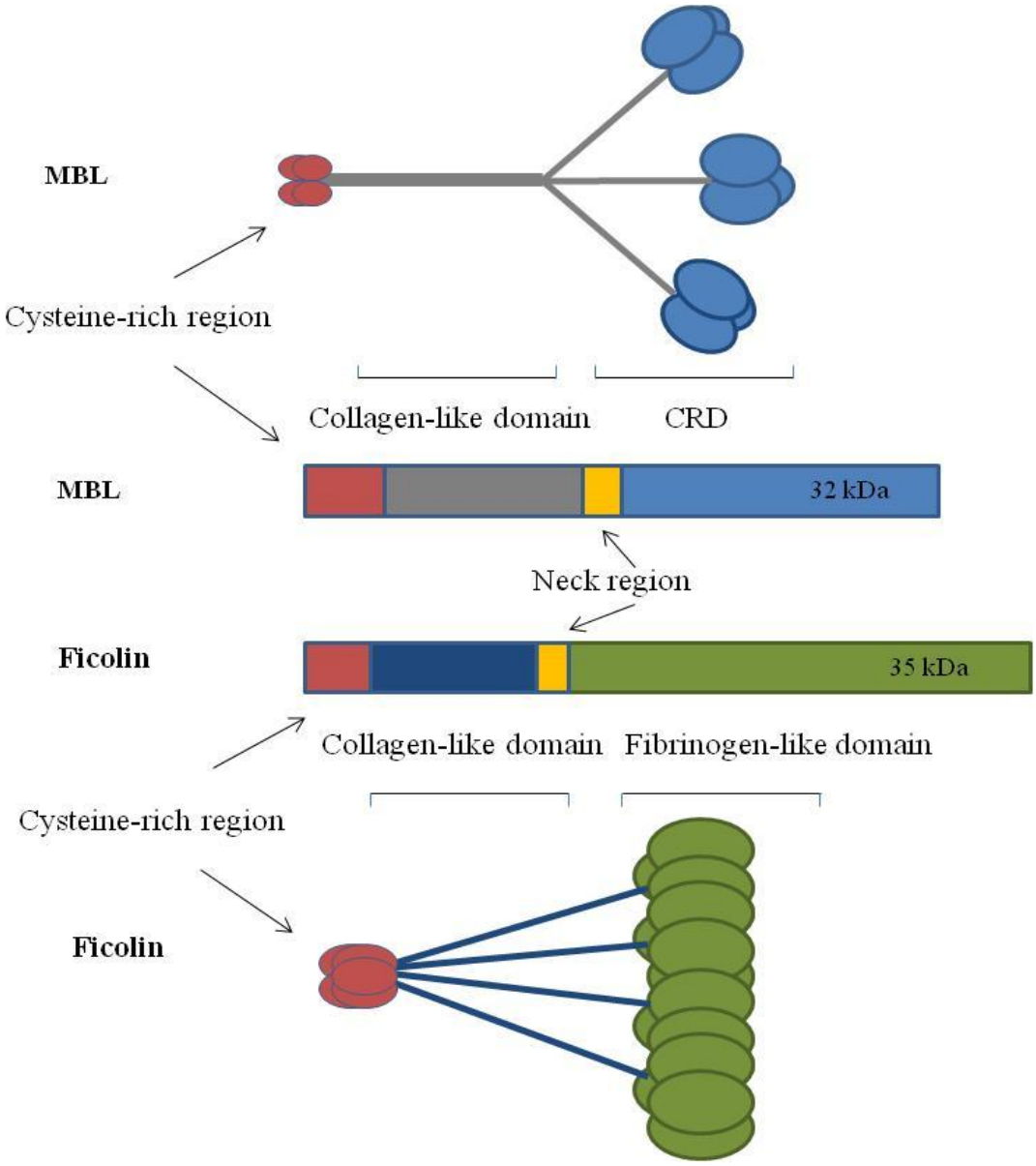


Figure 3. Domain and oligomeric structure of mannose-binding lectin and ficolins. The trimetric form of MBL and tetrameric form of L-ficolin that was indicated by electron microscopy are shown (Fujita, 2002)

1.3.3.3 MBL

Mannan-binding lectin (MBL, also known as mannose-binding protein) is a member of the collectin family and a plasma glycoprotein (Malhotra et al., 1992). The 32 kD polypeptide chain of MBL consists of 228 amino acids plus a 20 residue cysteine rich region after which there is the collagenous part followed by a neck region and

finally a C-terminal calcium-dependent lectin domain that binds carbohydrates (Presanis et al., 2003). Plasma complexes are found in various oligomeric forms. Between two to six structural subunits bind through the cystine-rich N-terminal to form structures ranging from dimers to hexamers (Teillet et al., 2005). Oligomerization of MBL molecules creates a compound which is able to have a strong binding to carbohydrate ligands due to the presence of multiple carbohydrate recognition domains, however, molecules with less polymerization have less avid carbohydrate binding capacity which results in reduced ability to activate complement (Wallis and Drickamer, 1999, Chen and Wallis, 2001).

The carbohydrate recognition domain of MBL binds to the 3-hydroxyl and 4-hydroxyl groups of sugars such as *N*-acetyl-D-glucosamine, mannose, *N*-acetyl-mannosamine, fucose and glucose in a calcium dependent manner (Shiratsuchi et al., 2008). MBL has been shown to bind IgM and activate the LP both *in vitro* and *in vivo* in myocardial and intestinal models of ischaemia reperfusion injury (McMullen et al., 2006, Busche et al., 2009). Binding of MBL to polymeric IgA followed by complement activation has also been demonstrated suggesting a role for MBL in mucosal immunity as well as in IgA nephropathy (Roos et al., 2001).

Only one form of MBL has been identified in human (Sastry et al., 1989), while in rodents MBL has two distinct forms, MBL-A and MBL-C (Sastry et al., 1995). Human MBL has structural and functional similarities to both forms of rodent MBL. Both forms of mouse MBL have been shown to activate the lectin complement pathway (Hansen et al., 2000).

The location of the human MBL gene has been identified on the long arm of chromosome 10 and analysis of the gene has demonstrated that the coding region contains four exons (separated by three introns) each of which is responsible for

encoding a distinct part of the protein (Sastry et al., 1989, Taylor et al., 1989). Two human MBL genes have been identified of which only MBL-2 is able to encode the MBL protein (MBL-1 is a pseudo-gene and doesn't produce a functional product)(Guo et al., 1998). Single point mutations in the MBL gene result in production of variant MBL alleles which do not have the functional capabilities of the normal variant (Sumiya et al., 1991). Three MBL gene polymorphisms have been described on codons 54, 57 and 52 of the first exon (which encodes the collagenous part of the MBL molecule) resulting in genotypes B, C and D respectively (Garred et al., 2003).

Several studies have investigated the presence of these variants in different populations and have shown their presence in up to 50% of healthy populations of various origins such as African, Caucasians, Asians and Eskimos (Madsen et al., 1998, Lipscombe et al., 1992, Garred et al., 1992).

Serum level of MBL in healthy humans is very variable and has been reported to be between 0 to more than 5 µg/ml (Presanis et al., 2003). MBL deficiency is shown to be associated with a lack of opsonisation capacity and recurrent infections in both children and adults (Super et al., 1989, Summerfield et al., 1995, Garred et al., 1995) although high serum MBL levels can increase the survival of certain intracellular pathogens such as mycobacterium tuberculosis or Trypanosoma cruzi by facilitating their entrance into the phagocytic cells (Kahn et al., 1996, Garred et al., 1997). These studies demonstrate that the role of MBL in protecting against infection varies depending on the pathogen and that high levels of MBL could increase the susceptibility of the host to certain infections.

1.3.3.3.1 MBL in kidney disease

In addition to its role in kidney IRI (Moller-Kristensen et al., 2005, de Vries et al., 2004), MBL has been identified to participate in the pathophysiology of various other kidney diseases.

One of the common kidney diseases associated with MBL is IgA nephropathy in which deposition of IgA and complement components in the kidney mesangium leads to glomerular damage. Matsuda et al have demonstrated the deposition of MBL and IgA in the glomeruli of a subgroup of patients with IgA nephropathy which has been associated with increased proteinuria and lower creatinine clearance (Matsuda et al., 1998). The association between MBL staining in the kidney tissue of patients with IgA nephropathy and disease severity was not present in two other studies (Endo et al., 1998, Lhotta et al., 1999) although in a later study consisting of 60 patients with IgA nephropathy, Roos et al showed mesangial deposition of MBL in a quarter of patients in addition to L-ficolin, MASP enzymes and C4d (Roos et al., 2006). They also showed that patients with MBL and L-ficolin deposition in their kidneys have evidence of worse histological kidney damage as well as significantly higher levels of proteinuria suggesting a role for the LP of complement in the pathogenesis of the more severe form of the disease.

Deposition of MBL has also been shown in cases of nephritis associated with Henoch Schönlein purpura (HSP), although the significance of this finding and its effect on complement activation and development of nephritis is controversial with one study finding no association between disease activity, the deposition of MBL and other complement factors in the kidney (Endo et al., 2000) and another showing a positive association between the two factors (Hisano et al., 2005). The number of patients in both of these studies were relatively small (10 and 31 respectively) and

perhaps larger studies would be able to clarify the real influence of MBL in the development of HSP.

Another kidney disease shown to be influenced by MBL levels is diabetic nephropathy. Several studies have shown a higher level of MBL in the serum of patients with Type 1 diabetes who develop albuminuria (Saraheimo et al., 2005, Hovind et al., 2005). Although the variation between the MBL levels in these patients has been related to MBL genotype in one study (Hansen et al., 2004), investigation of a large cohort (over a thousand patients) of type 1 diabetics by Kaunisto et al did not find a connection between various MBL genotypes and development of diabetic nephropathy (Kaunisto et al., 2009). Despite showing an association between serum MBL levels and proteinuria in diabetic patients, none of these studies have looked into the presence of MBL in the kidney of these patients which is of course difficult to do as there is often no indication for kidney biopsy in these patients. It does however remain unclear if the elevated level of MBL in patients with diabetic nephropathy is the cause of the kidney disease or the result of the metabolic disturbance in these patients.

The effect of MBL in kidney transplantation has also been the subject of several studies. Measurement of MBL levels in 266 renal allograft recipients revealed that patients with MBL levels above 400 ng/ml had worse graft survival in 10 years than the recipients with MBL levels of less than 400 ng/ml due to the higher incidence of transplant rejection which was resistant to treatment in the former group (Berger et al., 2005). The same group has investigated the relationship between MBL levels and kidney-pancreas graft survival in diabetic patients with simultaneous pancreas-kidney transplant (SPK). In a cohort of 99 patients with SPK after 12 years of follow up, they found that patients with high MBL levels (>400 ng/ml) had not only worse

kidney and pancreas graft survival but also higher rates of mortality related to cardiovascular death (Berger et al., 2007). The authors have suggested that higher MBL levels could lead to its increased binding to the damaged tissue and cause worsening of injury and enhance antigen presentation.

1.3.3.4 Ficolins

Ficolins were originally identified as transforming growth factor (TGF)-β1-binding proteins on porcine uterus membranes (Ichijo et al., 1993). In both human and mouse serum ficolin have been shown to have a binding specificity for N-acetylglucosamine (Fujita et al., 2004a). In humans, three types of ficolin have been identified: L-ficolin and H-ficolin that are present in the serum and M-ficolin which has been identified in leucocytes and lung but not in serum. Hepatocytes are the main source of L-ficolin and H-ficolin although the latter is also produced by bile duct epithelial cells as well as ciliated bronchial and type II alveolar epithelial cells in the lung (Runza et al., 2008). All three ficolins have been shown to activate the LP in association with MASPs (Liu et al., 2005). The ficolin ligands are mostly *N*-acetlyglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and complex-type oligosaccharide chains with GlcNAc residues present on pathogens (Runza et al., 2008). Unlike MBL, ficolins don't bind to mannose and lactose carbohydrates.

Two types of ficolins have been identified in mice named ficolin-A and ficolin-B. Expression of ficolin-A has been seen in liver and spleen (Fujimori et al., 1998). Ficolin-A is structurally similar to human L-ficolin and is present in plasma. Ficolin-B has 60% identity to ficolin-A and is expressed in bone marrow and spleen (Ohashi and Erickson, 1998). Although both ficolin A and B are able to bind to GlcNAc and

GalNAc, only ficolin A is able to bind to the MASP-2 enzyme and activate the LP (Endo et al., 2005).

1.3.3.5 Collectin 11

Collectin 11 (CL-11), also known as collectin kidney (CL-K1) is a member of the collectin family which has been recently cloned and characterized (Keshi et al., 2006). Keshi and colleagues have shown that CL-11 binds to sugars such as fucose and to a lesser extent mannose in a calcium dependent manner. Later, Hansen and colleagues demonstrated that in addition to being present in plasma at a concentration of about 2.1 µg/ml, organs such as kidneys, adrenal glands and liver are the main tissue sites of expression of CL-11 and more importantly, the molecule interacts with MASP-1 and/or MASP-3 in plasma (Hansen et al., 2010). They also showed that CL-11 can bind to the intact surface of various pathogens including bacteria, viruses and fungi suggesting an important role for this molecule in host defense. Recently CL-11 from both mouse and human has been shown to bind to the surface of Streptococcus pneumoniae and activate the lectin pathway of complement in association with MASP-2 independently of C4, emphasizing the importance of non-MBL members of the collectin family in the fight against pathogens (Ali et al., 2012).

1.3.3.6 MASPs

The Mannan-Binding Lectin-Associated Serine Proteases are proteolytic enzymes of the serine protease super family that are responsible for activation of the lectin pathway. They are homologous to the C1r and C1s of the classical pathway with similar domain organization (Schwaeble et al., 2002). Three MASPs have been identified so far, named MASP-1, MASP-2 and more recently MASP-3 (Dahl et al.,

2001). MASP-1, -2 and -3 are produced respectively as pro-enzymes of 91, 78 and 101.5 kDa. MASP-3 is an alternative-splicing product from the MASP-1 gene (on chromosome 3 in human) meaning that it carries the same heavy chain but has a different serine protease domain (Dahl et al., 2001). MAp19, an alternatively spliced product of the MASP-2 gene, is a small (19 kDa) non-enzymatic protein which is also associated with lectin–MASP complexes (Wallis, 2007, Stover et al., 1999b). The structural MASP-2 gene is located on chromosome 1 in humans and on chromosome 4 in the mouse (Stover et al., 1999b). The 3 MASPs and MAp19 have been found to be associated with MBLs and ficolins (Matsushita et al., 2000, Iwamoto et al., 2010).

Following the binding of MBL and ficolins to sugars or N-acetyl groups on pathogens or altered cells, the pro-enzyme form of MASP cleaves and the enzyme acquires proteolytic activities against complement components (McQuillin et al., 2009, Bornstein, 2008). MASP-1 has been shown to cleave C3 and C2 but not C4 (Matsushita et al., 2000, Hanley et al., 2012) and since its effect on C3 is very weak there is doubt if it has any biological significance (Gulbransen et al., 2012). Due to its ability to cleave C2, MASP-1 can potentially enhance the activation of complement that has been started by MASP-2/MBL complex although it is unable to initiate the activation (as it can't cleave C4) (Qu et al., 2011). More recently MASP-1 has been shown to be important in AP activation by cleaving factor D (Woehrle et al., 2010, Brough et al., 2009).

MASP-2 has been recognized as the key enzyme for LP activation, being able to cleave C2 and C4 leading to the assembly of C4b2a, the C3 convertase of the LP (Orellana et al., 2011). Although liver was described to be the only source of MASP-2 production (Stover et al., 1999a), a recent report has demonstrated its expression in

esophageal squamous cell carcinoma cells (Verma et al., 2006) raising the possibility that MASP-2 can be synthesized in other tissues in pathological conditions.

The functions of MASP-3 and MAp19 are not completely clear. In a recent study, Iwaki and colleagues have shown that in association with MBL, MASP-3 pro-enzyme becomes activated in the presence of bacteria and is able to initiate the activation of the AP and opsonisation of the bacteria by C3 (Iwaki et al., 2011). More studies are required to clarify the exact role of MASP-3.

1.3.4 The Terminal Pathway

One of the first well-defined functions of complement is the assembly of the complement components C5, C6, C7, C8 and C9 to form a membrane attack complex (MAC) which can create a pore in the lipid bi-layer of cell membrane and cause cell lysis.

Cleavage of C5 by C5 convertase produces C5b which binds to C6 molecule creating the C5b-6 complex that binds to C7. This reaction leads to a conformational change in C7 that result in a hydrophobic site of the molecule to be exposed and allow it to insert in the lipid bi-layer. C8 consists of three proteins, α, β and γ. The β unit of C8 binds the C5b-6-7 complex following which the C8α-γ binds to C9 and initiates the polymerization of several C9 molecules (10-16) into a pore-like structure with an internal diameter of 10 Å which is the MAC (DiScipio and Hugli, 1985, Hadders et al., 2012). The pore allows small molecules to equilibrate across the membrane which creates an osmotic imbalance due to retention of large molecules in the cell. This leads to an influx of water in the cell that causes cell swelling and ultimately lysis (Tschopp, 1984).

Although the lytic activity of the MAC is one of its well known functions, the complex has been shown to induce non lethal cell signals such as endothelial cell activation in xenotransplantation models (Dalmasso et al., 2000), the ability to induce the release of fibroblast growth factor and platelet-derived growth factor from endothelial cells, potentially leading to abnormal cell growth (Benzaquen et al., 1994) and interestingly to play a role in the pathophysiology of Alzheimer's disease (Webster et al., 1997).

1.4 Anaphylatoxins

The anaphylatoxins C3a, C4a and C5a are small (~10 KDa) peptides, containing 74-77 amino acids and are released after splitting of C3, C4 and C5 molecules respectively during complement activation. These peptides are not only potent inflammatory mediators but also play a significant role in regulating many aspects of the immune response (Klos et al., 2009). Control of anaphylatoxin activity is very important and is mediated by serum carboxypeptidase-N, which is able to remove the arginine (Arg) present at the C-terminal of these molecules leading to their inactivation (Bokisch and Muller-Eberhard, 1970).

C4a has been shown to be the weakest of the anaphylatoxins (Gorski et al., 1979) and most studies on anaphylatoxins have been concentrated on C3a and C5a. Of the well known functions of C3a and C5a are induction of smooth muscle contraction, release of histamine from basophils and mast cells, increase in small blood vessel permeability and chemo attraction of inflammatory cells (Klos et al., 2009).

More recently, the role of anaphylatoxins on regulation of adaptive immunity has been the subject of several studies. Use of C3a and C5a receptor deficient mice (C3aR-/- and C5aR-/- respectively) as well as C3a and C5a receptor antagonists has

facilitated our understanding of anaphylatoxins in adaptive immunity. Dendritic cells have been shown to produce C3a and C5a locally which following interaction with their G protein coupled receptors are able to enhance the expression of MHC class II and co-stimulatory molecules such as B7 and CD40 resulting in enhanced T cell response (Peng et al., 2009). DCs from C3aR-/- and C5aR-/- mice as well as wild type (WT) DCs treated with C3a or C5a receptor antagonist have not only less MHC class II and co-stimulatory molecule expression but also produce less IL-12 (which is required to stimulate IFN-γ production that leads to proliferation of Th1 cells) and more IL-10 (an anti-inflammatory cytokine that reduces Th1 response) resulting in an overall less efficient Th1 response (Peng et al., 2008, Li et al., 2008, Strainic et al., 2008). Importance of anaphylatoxins in T cell mediated response has been demonstrated in *in vivo* experiments as well. Mice deficient in C3a and C5a receptor (C3aR-/-/C5aR-/-) developed less infiltration of T cells in their cornea compared to WT following induction of herpes simplex keratitis and when infected with *Toxoplasma gondii*, died after a few days compared with the WT mice that survived due to their intact T cell function (Strainic et al., 2008). Testing this hypothesis in transplantation, Peng and colleagues showed that skin grafts from C3aR-/- mouse to allogeneic WT mouse had longer survival compared with WT to WT skin grafts as the C3aR-/- DCs in the donor skin were unable to stimulate the T cell response as effectively as the WT DCs (Peng et al., 2008). Influence of anaphylatoxin C5a on $CD8^+$ T cells has been investigated in another study where use of C5aR antagonist prevented mice infected with *influenza type A* virus to mount a flu-specific $CD8^+$ T cell response suggesting that engagement of C5a with its receptor has an effect on $CD8^+$ T cell activation (Kim et al., 2004). More recently Jörg Köhl's group have shown that activation of C5aR on DCs is an important step in formation of signals

that drive the differentiation of $CD4^+$ T cells to the regulatory, Th1 or Th17 subtype by change in cytokine production as well as co-stimulatory molecule expression (Weaver et al., 2010).

The influence of anaphylatoxins on the γδ T cells has also been the subject of a number of studies. Han G and colleagues have demonstrated the presence of C5aR on γδ T cells and have shown that in a mouse sepsis model (following cecal ligation and puncture), the expression of C5aR on these cells increases and engagement of C5a with its receptor on γδ T cells results in an increase in IL-17 production (Han et al., 2011). In an *Escherichia coli (E. coli)* induced mouse model of sepsis, activation of C5aR on NK and NKT cells was shown to result in an increase in production of IFN-γ and TNF-α and reduce the mice survival (Fusakio et al., 2011).

In the past decade, presence of anaphylatoxin receptors and their effect has been investigated on a variety of stem cells. Mesenchymal stem cells which have a significant potential for tissue regeneration and repair, have receptors for both anaphylatoxins C3a and C5a and are subject to chemoattraction by them to injured tissue (Schraufstatter et al., 2009). Presence of C3aR on the haematopoietic and progenitor cells has on the other hand been shown to be important in homing and retention of these cells in the bone marrow (Ratajczak et al., 2004). Rahpeymai and colleagues have demonstrated that both C3a and C5a receptors are expressed on neural stem cells and neural progenitor cells and that they positively regulate neurogenesis following cerebral ischaemia (Rahpeymai et al., 2006).

In brief, anaphylatoxins influence the adaptive immune response through various mechanisms such as direct effect on antigen presenting cells (i.e. dendritic cells and macrophages), resulting in modulation of their effect on T cells as well as inducing production of various inflammatory cytokines such as IFN-γ, IL-4, IL-6, TNF-α and

IL-12 family from tissue resident macrophages and epithelial cells which affects the differentiation and expansion of Th17 cells and has an influence on the balance of Th1 and Th2 cell populations (Zhou, 2012).

The effect of anaphylatoxins on B cells is less extensively studied, although they have been shown to influence their cytokine production and chemotaxis (Fischer and Hugli, 1997, Kupp et al., 1991). Investigating the role of anaphylatoxins in tissue repair is an important field for future research.

1.4.1 Anaphylatoxin receptors

There are three known anaphylatoxin receptors: C3a receptor (C3aR), C5a receptor (C5aR) and C5a receptor-like 2 (C5L2). They belong to the family of G-protein coupled receptors which are among the largest family of proteins of the mammalian genome (Takeda et al., 2002). They consist of seven trans-membrane helices with an extracellular N-terminus and an intracellular C-terminus (Kroeze et al., 2003). They have many structural similarities but differ in their ligand specificity and function.

Both C3a and C5a receptors are present on a variety of cells including dendritic cells, neutrophils, monocytes, macrophages, basophils and eosinophils. Some non-myeloid cells also express these receptors such as kidney tubular epithelial cells, endothelial cells, astrocytes, liver parenchymal cells and bronchial and alveolar epithelial cells (Ames et al., 1996, Wetsel, 1995, Zwirner et al., 1999, Ischenko et al., 1998, Zahedi et al., 2000, Braun et al., 2004).

Presence of the C3aR on T cells has been a matter of debate. Werfel et al have shown the presence of C3aR on activated T cells (Werfel et al., 2000) but they have not been detected on un-stimulated T or B cells (Martin et al., 1997). Presence of C5aR on T cells has been shown in several studies (Lalli et al., 2008, Strainic et al., 2008)

although Soruri and colleagues could not detect the expression of C5aR on murine lymphoid cells using anti-C5aR antibodies and another study using fluorescent labeled C5a showed binding by only 6% of lymphocytes (van Epps and Chenoweth, 1984).

The widespread presence of anaphylatoxin receptors and their broad range of functions has invited significant interest into the study of their biological functions and production of compounds that can block their effect and modulate their function.

1.4.2 Complement regulatory proteins

As complement activation has the potential to induce significant inflammation and tissue injury, its function needs to be tightly regulated to prevent host damage. This regulation is executed via several fluid phase and membrane bound proteins (Hourcade et al., 1989). Table 1 shows a list of complement regulatory proteins.

Component	Size (kD)	[Serum](μg/ml)	Site of action
Soluble proteins			
C1 inhibitor	105	200	C1r, C1s, MASPs
Factor I	88	35	C4b, C3b
Factor H	150	480	C3b
C4BP	500	250	C4b
Vitronectin (S-Protein)	83	505	C5b-7
Clusterin (SP-40)	70-80	50-100	C5b-9
Properdin	52-55	5-25	C3/C5 convertase
Membrane-bound proteins			
CR1 (CD35)	190-220	-	C3/C5 convertase
MCP (CD46)	45-70	-	C3/C5 convertase
DAF (CD55)	70	-	C3/C5 convertase
CD59	20	-	C5b-8/C5b-9

Table 1. Complement regulatory proteins. C4BP, C4b binding protein; CR1, complement receptor type 1; MCP, membrane cofactor protein; DAF, decay accelerating factor

1.4.2.1 Fluid phase regulatory proteins

A number of proteins act as complement regulators in the fluid phase. C1 inhibitor (C1INH) is an inhibitor of serine protease enzymes and regulates the activation of the complement system by deactivating C1r and C1s of the classical pathway and MASP-2 of the lectin pathway (Davis, 2004) thereby preventing the formation of C4/C2 convertase complex. Factor I, a very specific serine protease, is another fluid

phase complement regulator. It prevents the formation of both C3 and C5 convertases of the three complement pathways by cleaving the α-chains of C3b and C4b (Kavanagh et al., 2005). C4b-binding protein (C4BP) acts as a co-factor for serum factor I, enhancing the proteolytic inactivation of C3b and C4b (Blom et al., 2003). When bound to C4b, in addition to preventing the formation of the classical and lectin pathway C3 convertase, C4bC2a, it accelerates its natural degradation (Gigli et al., 1979). Factor H is an important negative regulator of the alternative pathway of complement. It acts as a co-factor for factor I in C3b cleavage and inactivation and enhances the degradation and decay of C3bBb which is the C3 convertase of the alternative pathway (Ferreira et al., 2010). Factor H also protects the host cells from the damaging effect of the alternative pathway activation. This is achieved by the high affinity of factor H to bind sialic acid residues present on host cells which allows factor H to cleave the C3b molecules that bind to the host cell surface and prevent the activation of the alternative pathway (Fearon, 1978) whereas pathogens that don't have the sialic acid residue on their surface remain sensitive to destruction by the alternative pathway activation. Clusterin (SP-40) and vitronectin (S protein) are inhibitors of the membrane attack complex formation. They bind to C5b-9 and make the complex water soluble, hence preventing it from insertion into cell membrane and induction of cell lysis (Chauhan and Moore, 2006). Carboxypeptidase-N is responsible for deactivation of the anaphylatoxins C3a, C4a and C5a by cleaving the arginine amino-acid from the C-terminal side of these peptides (Matthews et al., 2004).

1.4.2.2 Properdin

Louis Pillemer and his collaborators in the early 1950s described properdin as a molecule which was able to activate the complement pathway without involvement of an antigen-antibody complex (Pillemer et al., 1954). After the initial interest in this molecule, it was reported that properdin preparations contain antigen-antibody complexes and that the activation of the complement pathway is through the established classical pathway (Nelson, 1958). After recognition of the alternative pathway, attention to properdin was re-instated and several studies thereafter investigated its role in complement activation (Muller-Eberhard and Schreiber, 1980). Properdin exerts its effect as a positive regulator of the alternative complement pathway by binding to and stabilizing C3 and C5 convertase complexes, C3bBb and C3b(n)Bb (Fearon and Austen, 1975, Muller-Eberhard and Schreiber, 1980). Properdin has an important role in optimal activation of the alternative pathway and patients deficient in properdin have been shown to develop fatal septicaemia mostly due to meningococcal infection (Fijen et al., 1999).

Properdin is formed of small (53kD) identical protein subunits which bind head to tail to form dimers, trimers and tetramers (Smith et al., 1984). In contrast to most other complement proteins, properdin is not synthesized in the liver and its secretion has been shown from monocytes, $CD4^+$ and $CD8^+$ T cells and several bone marrow progenitor cell lines (Schwaeble et al., 1993, Schwaeble et al., 1994, Farries and Atkinson, 1989). Neutrophils are able to secrete properdin after stimulation with factors such as TNF-α, IL-8, C5a and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (Wirthmueller et al., 1997). Endothelial cells exposed to sheer stress have been implicated as another source of properdin (Bongrazio et al., 2003).

Interestingly, properdin has been shown to act as a pattern recognition molecule, binding to apoptotic T cells, thereby facilitating their phagocytosis via activation of the alternative pathway (Kemper et al., 2008).

1.4.2.3 Membrane bound regulatory proteins

Membrane bound regulatory proteins are expressed on various host cells and are responsible for protecting the host tissues from damage due to complement activation.

Complement receptor 1 (CR1, also known as CD35) is a transmembrane glycoprotein which accelerates the decay of C3 and C5 convertases of both classical and alternative complement pathways and also acts as a co-factor for factor I in degrading C3b and C4b molecules (Ahearn and Fearon, 1989). Membrane Cofactor Protein (MCP, also known as CD46) is another cofactor for factor I, assisting the cleavage of C3b and C4b (Liszewski et al., 1991). Decay accelerating factor (DAF, also known as CD55), inhibits formation of C4b2a and C3bBb, the C3 convertases of the classical/lectin and alternative pathways respectively by competitive inhibition of C2 and Factor B uptake (Kinoshita et al., 1986) as well as accelerating the decay of available C3 and C5 convertases (Medof et al., 1984). CD59 acts differently from the other membrane regulators. It prevents the formation of the membrane attack complex by inhibiting the binding of C9 to C5b-8 (Miwa and Song, 2001).

1.5 Biological functions of the complement system

Opsonization/phagocytosis, lysis, and generation of inflammatory response through soluble mediators known as anaphylatoxins to eliminate pathogens are well established biological functions of complement (Dunkelberger and Song, 2009). In addition, complement has been known to be responsible for clearance of immune

complexes (Walport and Davies, 1996). In the past decade, our understanding of complement and its functions has expanded and we know that complement is not only responsible as one of the first lines of defense against microbial infections but also plays a significant role in homeostasis by facilitating the removal of cellular debris and apoptotic cells, coordinating the immune response and acting on healthy cells that don't behave normally (Ricklin et al., 2010). Complement has also been found to be a key regulator of the adaptive immune response which is discussed in the next section.

Opsonization is a process by which certain complement molecules bind to pathogens or foreign particles and tag them so that they could be recognized by phagocytic cells such as neutrophils and macrophages. C3 is the main complement molecule involved in opsonization. Following activation, C3 is cleaved to form C3b which binds to Bb and forms the C3 convertase, C3bBb and cleaves C3 to produce more C3b. C3b is deposited on the target surface and is rapidly converted to inactive products including iC3b, C3c and C3dg which act as ligands for complement receptors 1-4 and CRIg on phagocytic cells. The interaction of the ligand and its receptor on phagocytic cells leads to clearance of the target by phagocytosis (Chen et al., 2007).

C3 has an important role in immune complex solubilisation and its removal from circulation (Schifferli, 1987). Immune complexes are formed constantly in the circulation and their levels increase significantly after exposure of host to antigen and initiation of a humoral immune response. The antigen-antibody complexes could be harmful when deposited on vascular endothelium as they can activate the classical complement pathway and lead to an inflammatory reaction and tissue damage. Covalent binding of complement factors C1, C4, C2, and C3 to immune complexes changes their biophysical characteristics and prevents their aggregation, so they

would remain soluble and do not precipitate (Schifferli et al., 1980, Hong et al., 1984). The immune complexes that have been opsonised with C3b would attach to cells that have the C3b receptor, CR1 (particularly on erythrocytes in the circulation), and are transferred to macrophages for elimination (Jensen, 2005).

MBL and C4 also act as opsonins, facilitating the uptake of various fungi, viruses and bacteria by polymorphonuclear cells (van Asbeck et al., 2008, Mascart-Lemone et al., 1983). Finally, C1q, which is a component of the classical pathway of complement has been shown to have an important role is opsonizing apoptotic cells from different tissues such as keratinocytes and vascular endothelial cells (Riley-Vargas et al., 2004), facilitating their removal in a physiological, non-inflammatory way. Elimination of apoptotic cells and immune complexes by C1q is an important feature, the absence of which leads to development of autoimmune diseases such as systemic lupus erythematosus (SLE) (Botto et al., 1998, Walport et al., 1998).

The lytic effect of complement is through formation of the MAC by assembly of C5b, C6, C7, C8 and C9 molecules which induces a pore in the lipid bi-layer of pathogens (Muller-Eberhard, 1986). Insertion of MAC complex in the cell membrane leads to an increase in intracellular Ca^{2+}, leakage of cellular nucleotide pools such as ATP and its precursors as well as loss of mitochondrial polarity, all of which result in cell death (Papadimitriou et al., 1994, Papadimitriou et al., 1991).

MAC has other biological functions beside its well known cytolytic effect such as induction of cell proliferation and initiating inflammation. MAC has been shown to induce activation of neutrophils and macrophages resulting in the production and release of inflammatory compounds such as prostaglandins, leukotrienes, thromboxanes and reactive oxygen metabolites (Hansch et al., 1984). In endothelial as well as glomerular mesangial cells, deposition of MAC has been shown to

stimulate the release of platelet derived growth factor and fibroblast growth factor leading to proliferation of vascular smooth muscle cells and glomerular mesangial cells which result in atherosclerosis and proliferative glomerular disease respectively (Niculescu and Rus, 1999, Lovett et al., 1987) .

As described in section 1.4, proteolytic cleavage of complement molecules C3, C4 and C5 through activation of C3 convertase, activated C1s and C5 convertase respectively, leading to generation of the small (74-78 amino acid) pro-inflammatory by-products C3a, C4a and C5a which are known as anaphylatoxins (Hugli, 1984). C3a and C5a in particular have several biological activities such as: smooth muscle cell contraction; release of histamine and serotonin from basophils and mast cells; increase in vascular permeability; promoting adhesion of leukocytes to endothelium and initiating the chemotaxis of inflammatory leukocytes (Kohl, 2001). Anaphylatoxin receptors are found on a variety of non-immune cells such as the heart, kidney, lung, liver, endothelial cells, neurons and astrocytes (Klos et al., 2009) suggesting that they modulate the function of these organs in health and disease. Anaphylatoxins also exert a significant effect on the adaptive immune response, as will be discussed below.

1.5.1 Production of complement proteins

With the exception of C1q, C7, and factor D, liver is the main source of production of circulating complement proteins, although a number of studies in the past three decades have shown various extra-hepatic sources for synthesis of several soluble complement proteins with or without the presence of an external stimulus (Morgan and Gasque, 1997). Monocytes, macrophages, dendritic cells, fibroblasts and endothelial cells have all been shown to produce some of the complement proteins

(Perlmutter and Colten, 1986). Organs such as the brain and the kidney are also capable of producing several of the complement proteins suggesting the importance of these molecules in dealing with potential local infection and tissue injury (Chen, 2004, Geissmann et al., 2010, Dodds, 2002, Gordon, 2002) although they might actually contribute to local inflammation (Lawrence and Natoli, 2011).

C1q is mainly produced by monocytes, macrophages, immature dendritic cells and microglial cells of the brain (Mantovani et al., 2009, Ji et al., 2012, Zhang et al., 2010, Martinez et al., 2008, Le Berre et al., 2005). Production of C1q by these cells could have an important role in the clearance of local immune complexes and apoptotic cells (Mantovani et al., 2004). Monocytes and tissue macrophages such as liver kupffer cells as well as fibroblasts are known sources of C7 (Lawrence and Natoli, 2011, Mantovani et al., 2009). Factor D is produced by adipocytes suggesting a role for complement in lipid metabolism (Mantovani et al., 2004).

1.6 Role of complement in adaptive immunity

1.6.1 Complement factors and humoral immunity

The involvement of the complement system in humoral immunity has been recognized since the late 1960s when Gajl-Peczalska and colleagues detected the presence of both C3 and complement fixing immune complexes in the germinal centre of lymphoid follicles suggesting the existence of a relationship between complement and B cell activation (Gajl-Peczalska et al., 1969) followed later by recognition of complement receptors on some lymphoid cells (Bianco et al., 1970). It was shown later that depletion of complement C3 impairs antibody responses (Pepys, 1976), further implying a role for complement in adaptive immunity.

It took several years to realize that complement components influence the humoral immune response by affecting the B cells at various stages of their development (Carroll, 2004). CR1 and CR2 (also known as CD21) which are expressed on follicular dendritic cells (FDCs) and B cells play an important role in regulating the humoral response (Fang et al., 1998). The signaling protein CD19 and CD81 protein join CR2 to form what is known as the B-cell co-receptor complex. Carter and Fearon demonstrated that engagement of complement-coated antigens with this B cell co-receptor, CD21/CD19/CD81, enhances positive selection of B cells (Carter and Fearon, 1992). Immunizing mice with hen egg lysosome using C3d as an adjuvant, Fearon's group later showed that C3d was able to significantly enhance the antibody production (Dempsey et al., 1996).

In addition to their role in humoral immune response, complement receptors CD21 and CD35 help with B cell survival in the germinal centre by inducing an antigen-independent signal (Fischer et al., 1998, Carroll, 1999).

The role of anaphylatoxins C3a and C5a on the B cell response has also been the subject of investigation in several studies. Although C3aR is not expressed on all B cells, its presence has been demonstrated on tonsillar B cells, and stimulation of these cells with C3a and C3a-desArg has been shown to suppress their polyclonal antibody response as well as reduce their production of IL-6 and TNF-α in a dose dependent manner (Fischer and Hugli, 1997). C5a on the other hand has been shown to have an effect on migration and trafficking of tonsillar naive and memory B cells as well as B cells of the germinal centre (Ottonello et al., 1999, Kupp et al., 1991).

1.6.1.1 Complement factors and T-cell mediated immunity

Mice with various complement component deficiencies have been used to examine the effect of complement in T-cell mediated immunity.

Being the centre of the three activation pathways of complement, C3 not only contributes to the well established pro-inflammatory effects of complement such as opsonization and anaphylatoxic activity, but also has an important role in immune regulation (Kinoshita, 1991). In a model of influenza virus infection, Kopf and colleagues showed that C3-/- mice had higher virus titers in their lung due to its delayed clearance, as well as reduced number of virus specific $CD4^+$ and $CD8^+$ effector T cells. In addition, proliferation of $CD4^+$ T cells and lytic activity of cytotoxic T cells from mediastinal lymph nodes of C3-/- animals was significantly reduced compared to wild type mice (Kopf et al., 2002). This mouse phenotype also had reduced $CD4^+$ dependent IgG response, suggesting that C3 or products of complement activation further down the pathway have a role in T cell priming in lymphoid organs as well as T cell-mediated antibody production.

Using a kidney transplant model in which WT or C3-/- kidneys were transplanted into congenic mice, Pratt and colleagues demonstrated that C3-/- transplants survive considerably longer than the WT (Pratt et al., 2002). They have shown that this effect is partly due to the fact that C3-/- tubular epithelial cells were less able to stimulate antigen-primed alloreactive T cells. This finding suggests that C3 has an important role in antigen presentation by tubular epithelial cells which had already been proposed (Kelley and Singer, 1993, Hagerty and Allen, 1992). Later, Li and colleagues confirmed the above results by showing in an *in vitro* model that C3 coated tubular epithelial cells were able to induce an allospecific T cell response and affect the production of T cell specific cytokines (Li et al., 2004). They also showed

that antigen presenting cell expression of complement was an important co-stimulatory factor for the priming of alloreactive T cells in mice (Zhou et al., 2006, Peng et al., 2008, Sacks and Zhou, 2012).

Following the above studies, to investigate the potential role of anaphylatoxins in T-cell response, Kim and colleagues used a C5aR antagonist in a mouse model of *influenza A* infection. They demonstrated a significant reduction in the number, cytotoxic ability and INF-γ production of $CD8^+$ T cells in the lungs of mice treated with C5aR antagonist compared with the mice treated with a control peptide indicating a role for C5a in $CD8^+$ T cell activation (Kim et al., 2004).

Anaphylatoxin C3a has also been shown to affect T cell function through its interaction with dendritic cells (DCs). Peng and colleagues have shown that DCs from C3aR-/- mice and wild type DCs treated with C3aR antagonist have a reduced capacity to induce a T cell response towards their expressed alloantigen (Peng et al., 2008). They noted that both C3aR-/- DCs and DCs treated with C3aR antagonist expressed fewer MHC class II and co-stimulatory molecules such as CD40, B7.1 and B7.2, which potentially explains the reduced T cell response (as optimal T cell activation is dependent on the co-stimulatory signals). Strainic and colleagues consequently demonstrated that both C3a and C5a and interaction of these anaphylatoxins with their respective receptors have an important role in expression of MHC class II and co-stimulatory molecules on DCs and consequently on T cell differentiation and proliferation (Strainic et al., 2008). They suggested that anaphylatoxins have a direct effect on T cells via their receptors although as discussed earlier, the presence of these receptors on T cells has been controversial.

Complement regulatory molecules have also been shown to modulate T cell response. Daf-/- mice have been shown to have an enhanced T cell response to active

immunization, secreting increased amounts of pro-inflammatory cytokines, IL-2 and INF-γ, and reduced amounts of anti-inflammatory cytokine, IL-10. This effect was complement-dependent as absence of either C3 or C5 abolished the increased T cell response in daf-/- mice (Fang et al., 2011). Heeger and colleagues have demonstrated that the increased T cell proliferation and frequency in daf-/- animals in response to antigen presentation (by daf-/- antigen presenting cells) is factor D and to some extent C5 dependent suggesting that the alternative pathway activation is important in influencing the T cell response (Heeger, 2005). These results suggest that daf molecule has an effect on T cell activation and potentially suppresses T cell response through inhibition of convertase activity leading to reduced alternative pathway activation and production of anaphylatoxins.

CD46, the complement regulatory protein which acts as a receptor for C3b, has been shown to influence T cell function in various ways. Astier and colleagues demonstrated that stimulation of CD46 on T cells not only resulted in tyrosine phosphorylation of complexes in the cell which regulate T cell receptor signaling but also in conjunction with CD3, it induced a co-stimulatory effect leading to increased T cell proliferation (Astier et al., 2000). Later, it was shown that stimulation of CD3 and CD46 on $CD4^+$ T cells in the presence of IL-2, induces a regulatory phenotype in these cells characterized by IL-10 production and suppression of T-helper cell activation (Kemper et al., 2003). Failure to switch from $CD4^+$ T-helper cell to the regulatory phenotype through CD46 activation has been shown to contribute to autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (Astier et al., 2006, Cardone et al., 2010).

1.7 The role of complement in IRI

The pathophysiology of the inflammatory cascade that is activated following the reperfusion of ischaemic tissue has been the subject of intensive research, as it is believed that modulation of this response in the early phase could prevent organ damage. Complement is one of the key contributors to the inflammatory response following IRI. Complement proteins and their active breakdown products participate in various aspects of the inflammatory response; They are able to initiate chemotaxis of leukocytes, stimulate the release of cytokines and cause vasodilatation as well as have an effect on the function of the coagulation pathway (Frank and Fries, 1991, Prydz et al., 1977).

In the early 1970s, Hill and Ward demonstrated the presence of C3 cleavage products associated with leukocyte infiltration in the damaged heart tissue of rats after ligation of their coronary arteries, suggesting for the first time that complement has an active role in inducing cellular damage following IRI (Hill and Ward, 1971). Following this finding, many studies using knock out animal models or complement inhibitors have shown the pathogenic role of complement products in IRI of different organs such as kidney, intestine and hind limb (Diepenhorst et al., 2009).

Investigations on the role of complement in IRI have shown that components from each complement activation pathway could be important in the damage caused depending on the involved organ for example the classical pathway has been shown to be important in intestinal (Schenk et al., 2008) and liver (Heijnen et al., 2006) IRI but not in the kidney. Although there was a well defined distinction between the effects of each pathway in IRI, the more recent studies raise the possibility of an interaction between the classical and lectin pathway activation. Furthermore, it is

possible that activation of either of these pathways act as an initial stimulus for alternative pathway activation which then continues its positive feedback loop.

As shown in Fig 2, C3 is the central component that is in common with all complement activation pathways and most of the recognized effector mechanisms depend on it. The effector molecules produced after C3 is shared in the all 3 pathways.

Cobra venom factor (CVF) was one of the first agents used in early *in vivo* complement studies. As its name implies, CVF is derived from cobra venom; it has structural similarity to C3 and functions like C3b, forming C3 and C5 convertase with longer half life than C3bBb leading to continuous conversion of C3 and C5 molecules and depletion of serum complement (Fritzinger et al., 2009). Maroko and colleagues in the late 70s showed that use of CVF in dogs who underwent cardiac ischaemia by occlusion of their left anterior descending coronary artery, developed less myocardial injury compared to a control group (Maroko et al., 1978). Complement depletion using CVF was later shown to reduce the infiltration of polymorphonuclear cells and reperfusion injury in post ischaemic rat livers (Jaeschke et al., 1993).

Considering that IgG and IgM are important activators of the classical pathway, recombinase activating gene-1 deficient (RAG-1-/-) mice which are unable to produce immunoglobulins due to the lack of mature B and T cells as well as complement receptor CD35 deficient (CR2-/-) mice have been used in studies investigating the role of the classical pathway in IRI. Use of these animals in the heart, hind limb and intestinal IRI models have shown that they have reduced level of tissue damage in the injured organ compared to their WT control (Williams, 1998, Weiser et al., 1996, Zhang et al., 2006a). A specific natural IgM directed towards a

self-antigen which is exposed by ischaemia seems to be responsible for inducing injury (Zhang et al., 2004, Chan et al., 2006b). Re-constitution of these animals with natural IgM restores their organ damage following IRI suggesting that IgM has an important role in activating the complement pathway thereby inducing injury. RAG-1-/- mice are not protected from kidney IRI (Park et al., 2002), indicating that IgM is not a major player in this model which emphasizes the fact that complement activation in IRI varies in different organs.

Interestingly, while absence of C1q has not provided any protective effect in myocardial and intestinal IRI, the activation of the lectin pathway via MBL has been shown to have an important role in inducing tissue damage in these organs demonstrated by the fact that mice deficient in MBL have less injury (Walsh et al., 2005, Hart et al., 2005). Previous experiments displaying the binding of IgM to MBL (Nevens et al., 1992, Arnold et al., 2005) raised the possibility that it is actually the lectin pathway that plays the key role in IRI and indeed later studies showed that in intestinal and myocardial IRI, tissue damage is dependent on the presence of both IgM and MBL (McMullen et al., 2006, Busche et al., 2009). Deficiency of C1q was not protective in these models suggesting that the effect of IgM was independent of the classical pathway (Zhang et al., 2006b). By showing that MBL binds to IgM molecules targeted towards a self non-muscle myosin heavy chain in murine models of intestinal and skeletal IRI (Zhang et al., 2006a), Zhang and colleagues strengthened the notion that LP plays the key role in IRI.

Kidney IRI has been shown to be independent of C4 which is a component of the classical as well as the lectin complement pathway (Zhou et al., 2000); However, the influence of the lectin pathway in kidney IRI has been demonstrated by Moller-Kristensen and colleagues where mice deficient in MBL A and C had 35% protection

from injury following IR compared to the WT mice (Moller-Kristensen et al., 2005). The importance of MBL molecules in this model was confirmed by reconstitution of these deficient mice with recombinant MBL-A and MBL-C after which they developed similar level of kidney injury as WT. Demonstration of MBL deposition in ischaemic kidney tissue of both mice and human has further indicated the importance of the LP in IRI (de Vries et al., 2004).

The effect of the LP has been shown in a mouse model of cerebral IRI where MBL null mice had smaller infarct size compared to the WT which was translated in a population of stroke patients, where genotypes with low MBL levels also showed better outcome in 3 months compared to the MBL sufficient group (Osthoff et al., 2011, Cervera et al., 2010). IRI in MBL deficient mice has shown to induce less damage in skeletal muscle (Chan et al., 2006a) and less infarct size in the heart with better preservation of left ventricular ejection fraction (Walsh et al., 2005). Use of monoclonal antibodies against MBL has been shown to reduce myocardial IR damage significantly in a rat model (Jordan et al., 2001). Most recently Schwaeble and colleagues have demonstrated that absence of LP activation in MASP-2-/- mice as well as treatment of wild type mice with anti-MASP-2 antibody protects them from cardiac and intestinal IRI (Schwaeble et al., 2011).

Investigations on the AP including use of factor B-deficient mice as well as utilization of an inhibitory monoclonal antibody to mouse factor B (FB) in a native kidney IR model have demonstrated a protective effect (Thurman et al., 2005, Thurman and Holers, 2006). Factor D-deficient mice have been used in a gastro-intestinal IR model where in comparison with the WT, they have been shown to developed significantly less injury demonstrated by less C3 deposition (Stahl et al., 2003). Furthermore, reconstitution of the factor D-deficient mice with recombinant

factor D in this experiment, resulted in development of injury comparable to the WT suggesting that factor D and hence the AP activation is responsible for induction of the damage.

A major outcome of animal IR studies was the finding that local production of complement C3 by kidney epithelial cells plays an important role in graft injury as experiments performed in mouse isograft transplant models in the absence of locally produced C3 have shown a remarkable reduction in reperfusion graft damage (Farrar et al., 2006). This study showed for the first time that local production of complement is an important factor in tissue damage following IRI.

The role of effector molecules which follow C3 activation have also been investigated in animal IRI models. Use of an antagonist against the anaphylatoxin C5a has shown a protective effect in several models of IRI including native kidney IR in mice as well as intestinal IR in rats suggesting the role of this molecule and potentially the downstream effector component C5b-9 in tissue injury (de Vries et al., 2003a, Arumugam et al., 2002). Administration of anti-C5 monoclonal antibody has also shown to have a protective effect in the mouse intestine and kidney as well as the rat heart following IRI (de Vries et al., 2003b, Wada et al., 2001, Vakeva et al., 1998). Absence of the terminal pathway complex, C5b-9 has also been shown to be protective from native kidney IRI (Zhou et al., 2000).

While progress has been made in identifying some of the effector mechanisms by which complement stimulates the genesis of IRI, there is more uncertainty regarding the molecular and biochemical mechanisms that initiate the complement mediated injury, i.e. which DAMPs are involved and how they trigger the activation of complement molecules that result in tissue injury. Table 2 summarizes the studies which have investigated the role complement molecules in mouse kidney IRI.

Complement Factor Investigated	Model of kidney IR	Author
C3, C4, C5, C6	Native mouse IR Using C3, C4, C5 and C6 deficient animals	Zhou et al., 2000
C5a	Native mouse IR Using Anti-C5 compound (preventing C5a formation)	de Vries et al., 2003
FB	Native mouse IR Using FB-/- animal and anti-FB monoclonal antibody	(Thurman et al., 2005, Thurman and Holers, 2006, Thurman et al., 2003)
MBL-A/MBL-C in mice MBL in human	WT Native mouse IR, Transplanted human kidney	(de Vries et al., 2004)
MBL-A/MBL-C	Native mouse IR Using double deficient animals	Moller-Kristensen et al., 2005
C3	Isograft mouse Transplant Using C3-/- animal	Farrar et al., 2006

Table 2. Summary of studies looking at the role of various complement components in native or transplant mouse model of kidney IRI

Knowing that anaphylatoxin C5a is an important mediator of IRI in the kidney and the fact that the potential role of C3a was unexplored in this model, I planned to first investigate the influence of this molecule in kidney IRI. At the same time, since there were increasing evidence that the lectin pathway of complement is the key trigger in IRI in various organs including the kidney, and due to the availability of MASP-2 deficient mice (from the laboratory of my second supervisor, Professor Schwaeble in Leicester) which have no lectin pathway activation (compared to MBL deficient mice which can still activate the lectin pathway via ficolins), I investigated the role of the lectin pathway in a kidney transplant model of IRI, compared to the effect of classical and alternative pathway activation using C4-/- and FB-/- mice respectively.

Therefore, the first two questions of my research were: a) to examine the expectation that C3a is a possible effector of IRI in the kidney and if so by what mechanism it induces injury and b) to address the hypothesis that the lectin pathway is a mediator of kidney IRI and specifically that MASP-2 deficient mice would be protected compared with C4 and FB deficient mice lacking the activation of the classical and alternative pathway respectively.

2 Materials and methods

2.1 Materials

General chemicals were purchased from Sigma-Aldrich or BDH (VWR, Leicestershire, UK). Histological stains were purchased from Raymond A. Lamb (London, UK). Microscope slides and solutions used for mounting were purchased from BDH (VWR, Leicestershire, UK).

2.1.1 Surgical Equipment

Clamp applicator and microaneurysm clamps were purchased from Codman (Berkshire, UK). Other surgical tools, including scissors, forceps, retractor and needle holders were purchased from Braun (Sheffield, UK). Ethicon sutures 4-0 were purchased from Southern Syringe Services (London, UK). Warm pads to keep even core body temperature in the animal during surgical procedures were purchased from VetTech (Cheshire, UK).

2.1.2 Antibodies

Rabbit anti-human C3d for tissue staining was purchased from DAKO (Cambridge, UK). FITC-conjugated goat anti-rabbit IgG from Jackson Laboratories (Maine, USA)

was used as secondary antibody for C3d staining. Rat anti-mouse anti-MASP-2 antibody was produced by Dr Youssif M. Ali (at Professor Schwaeble's lab) and kindly donated by Professor Schwaeble (Schwaeble et al., 2011) and goat anti-rat FITC antibody from Jackson laboratories (Maine, USA) was used as secondary antibody. Anti-MASP-2 antibody and its isotype control for the therapeutic experiments were provided by Omeros corporation (made by AbD Serotec, Düsseldorf, Germany).

2.1.3 Buffers and solutions

Phosphate Buffered Saline (PBS): PBS 10 x tablets purchased from Thermo Scientific (Hampshire, UK). 10 tablets were diluted in 1 liter of distilled water and autoclaved.

Lysis buffer:100 mM Tris-HCl, 20 mM EDTA, 1% SDS, 200 mM NaCl, pH: 8.

5 x TBE: 54 g Tris-Base, 27.5 g Boric acid, 20 ml of 0.5 M EDTA, pH: 8.

2.1.4 Mice

All mice used for experiments (WT and gene knock out) were on C57/BL6 (B6) background. WT mice were purchased from Harlan UK Ltd (Bicester, UK). C3aR-/- mice and MASP-2-/- mice were kindly donated by Professor Carroll from Harvard (Boston) and Professor Schwaeble from Leicester, respectively. MASP-2-/- mice were produced using a gene-disrupting construct (Stover et al., 1999b). C4 deficient (C4-/-) mice which were derived by homologous recombination in embryonic stem cells (Fredholm, 1997) were also obtained from Professor Carroll's group in Boston. C4-/-/MASP-2-/- double knockout mice were generated by back crossing the 2 strains for 10 generations in our department. Factor B deficient mice were a gift from Professor Stahl (Boston).

For native kidney IR experiments, male mice were used, and for transplantation, due to technical reasons, female mice were used throughout. Mice were 7-10 weeks of age and 19-23 grams of weight at the time of procedure. All mice were housed in pathogen free conditions at KCL facilities. All experiments were performed according to KCL and national guidelines and under Home Office Project Licence PPL70/6835.

2.2 Methods

2.2.1 Genotyping

All genetically altered mice were genotyped by a member of the research group after their arrival at KCL animal facilities and at regular intervals afterwards. I genotyped the C3aR-/- mice and the rest of the mice used in my thesis experiments were genotyped by Dr Anthony Farrer. DNA for genotyping was obtained from a portion of the mouse tail. WT mouse tail was used as control.

2.2.1.1 Preparing the genomic DNA

3-4 mm of mouse tail from WT and C3aR-/- mouse was collected, finely chopped and stored in a 1.5 ml eppendorf tube. 700 µl of lysis buffer was added followed by 35 µl of proteinase K (from a 10 mg/ml stock) and the tubes were then incubated at 55^{o}C overnight. After the overnight incubation, 25 µl RNase was added to the tubes and they were incubated for 2 hours at 37^{o}C. Following this and addition of 700 µl of Phenol-Chloroform-Isoamyl alcohol mixture (pH 8.0), the tube was vortexed for 5 minutes to mix the components well and then centrifuged at 14K for 10 minutes.

The aqueous phase that was separated on the top was collected in a fresh 1.5 ml eppendorf tube. 700 µl isopropanol was then added and the tube was inverted about

20 times until stringy precipitates formed. The tube was centrifuged at 14k for 5 minutes and the liquid was discarded, leaving a pellet at the bottom of the tube.

The pellet was washed with 75% ethanol followed by another wash with 100% ethanol. The ethanol was then discarded and the pellet was left on the bench to dry for 15 minutes. The pellet, containing genomic DNA, was then dissolved in 100 μl of nuclease-free water and stored at 4^{o}C, ready for use. 1 in 10 and 1 in 50 dilutions were used for genotyping assays.

DNA quantity was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.2.1.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was used on the genomic DNA to amplify the gene of interest and compare its expression in WT and gene knock out animal. Comparison was made with the presence of GAPDH gene. For the PCR reactions, 10-20 ng (4 μl) of the genomic DNA was used in addition to 5x Buffer, dNTP, Taq polymerase (all from Promega) and primers (from Sigma-Aldrich), bringing the total reaction volume to 12 μl as shown in Table 3 (F stands for forward and R for reverse).

Reaction Components	Volume per reaction (µl)	Final Concentration
5x Buffer (with 7.5 mM $MgCl_2$)	2.40	1 x
10 mM dNTP	0.24	0.2 mM
10 µM GAPDH-F	0.6	0.5 µM
10 µM GAPDH-R	0.6	0.5 µM
10 µM C3aRg-F (primer 1)	0.6	0.5 µM
10 µM C3aRg-R (primer 2)	0.6	0.5 µM
5 U/µl Taq Pol.	0.06	0.025 U/µl
H_20	2.9	
DNA (4 µl per reaction)	4.00	
Final volume	12.00	

Table 3. Components of the reaction mix and their volume for PCR experiments

After mixing the samples by gentle flicking, they were centrifuged for a few seconds. Applied Biosystems Gene Amp PCR system 9700 was used for gene amplification, at the condition described below:

1- 5 minutes at 95°C for denaturing of the DNA

2- 30 seconds at 94°C, denaturing

3- 45 seconds at 62°C, annealing of primers

4- 60 seconds at 72°C, extension

Repeat of steps 2-4, 35 times for amplification

5- 72°C for 10 min

The product was then cooled and kept at 4°C until analyzed. Table 4 shows the sequences for the primers used for genotyping the mice.

Genotype	Primer sequence	PCR conditions	Expected size (bp)
C3aR-/-	C3aR F 5'-CAGGTCAGCTCCTTCCTCTG-3' C3aR R 5'-AAAGCGGCGTTCAGTGT-3' GAPDH F 5'- ACCACAGTCCATGCCATCAC-3' GAPDH R 5'- CACCACCCTGTTGCTGTAGCC-3'	1. 95°C 5 min 2. 94°C 30s 3. 62°C 45s 4. 72°C 60s 5. repeat 2-4 x35 6. 72°C 10 min	WT 430 bp C3aR-/-230 bp
MASP-2-/-	MASP-2 F 5'-CATCTATCCAAGTTCCTCAGA-3' MASP-2 R 5'AGCTGTAGTTGTCATTTGCTTGA-3' MASP-2 Neo 5'-CTGATCAGCCTCGACTGTGC-3'	1. 95°C 5 min 2. 95°C 30s 3. 60°C 30s 4. 72°C 15s 5. repeat 2-4 x35 6. 72°C 10 min	WT 800 bp MASP-2-/- 500 bp
C4-/-	C4 F 5'-GCATTTCTCTCCCTTCTAGAACA-3' C4 R 5'-TGTAGCCCGTGGGTCTTAAG-3' C4 Neo 5'-CCTTCTTGACGAGTTCTTCTGA-3'	1. 95°C 5 mins 2. 94°C 30s 3. 62°C 30s 4. 72°C 15s 5. repeat 2-4 x35 6. 72°C 2 min	WT 600 bp C4-/- 458 bp
FB-/-	FB F 5'-GCAATACGCTGCCCACGACCGCAG-3' FB R 5'-CTTCTCAATCAAGTTGGTGAGGCACCG CTT-3' FB Neo 5'-TGCGTGCAATCCATCTTGTTCAAT-3'	1. 94°C 3 min 2. 94°C 30s 3. 67°C 1 min 4. 72°C 1 min 5. repeat 2-4 x35 6. 72°C 2 min	WT 600 bp FB-/- 928 bp

Table 4. PCR primer sequences and conditions for genotyping C3aR-/-, MASP-2-/-, C4-/- and FB-/-

2.2.1.3 Gel Electrophoresis

The gel required for electrophoresis was prepared by dissolving 1% agarose in 1x TBE by heating in a microwave and addition of 0.5 μg/ml of ethidium bromide. After cooling of the gel to room temperature, it was put in the gel tank which was filled with 1x TBE. The PCR products were loaded onto the gel and a 100 bp DNA ladder was used as a marker for molecular weight of the amplification products. The samples were run for 45 min at 90 volts. After completion of electrophoresis, the separated DNA fragments were viewed in Uvitec, a device using UV light to illuminate the fluorescence of the identified bands and the image was created with Xplorer software.

2.3 Induction of native kidney IRI

Male WT and C3aR-/- mice weighing between 19-23 grams were anesthetized by inhalation of isoflurane (Abbott Laboratories, Ltd, Kent, UK). Isoflurane was chosen as both induction and recovery from Isoflurane-induced anaesthesia is rapid, it has minimal side effects and is also relatively easy to titrate. Isofloran was administered mixed with approximately 0.4 litres of oxygen via a small mask and the flow was set constant throughout the procedure. The animal was placed on a warm pad throughout the procedure to maintain a constant body temperature. After the animal was completely anesthetized, a midline abdominal incision was made and the body cavity was held open with a pair of retractors. The connective tissue around the renal vein and artery of both right and left kidneys were gently cleared with a small forceps, and both vessels were clamped on each side for 50 minutes with a microaneurysm clamp. This period of ischaemia was used based on a previous study performed by Dr Zhou in our laboratory (Zhou et al., 2000). To confirm the suitability of this time point for my experiment, several experiments using various ischaemic times (40, 45 and 55 minutes) as well as

reperfusion times (2h, 4h, 12h and 48h) were performed and it was found that 50 minutes of ischaemia induced consistent injury with less than 5 percent mortality rate at 24 hours. After occlusion of the vessels, the abdominal wall was closed for the period of ischaemia. The clamps were then removed and kidneys observed for change in colour after reperfusion (successful clamping causes the kidney to go dark red in colour and reperfusion brings the normal dark pink colour back to the kidney). At this point 0.4 mls of warm saline was placed in the abdominal cavity and the incision was sutured. The animals were kept in warm incubator (28°C) for the first few hours after the procedure. They were sacrificed at 24 hours and blood samples were obtained using cardiac puncture following which the kidneys were removed for histological examination (both paraffin embedded and snap frozen samples were prepared)(Figure 4).

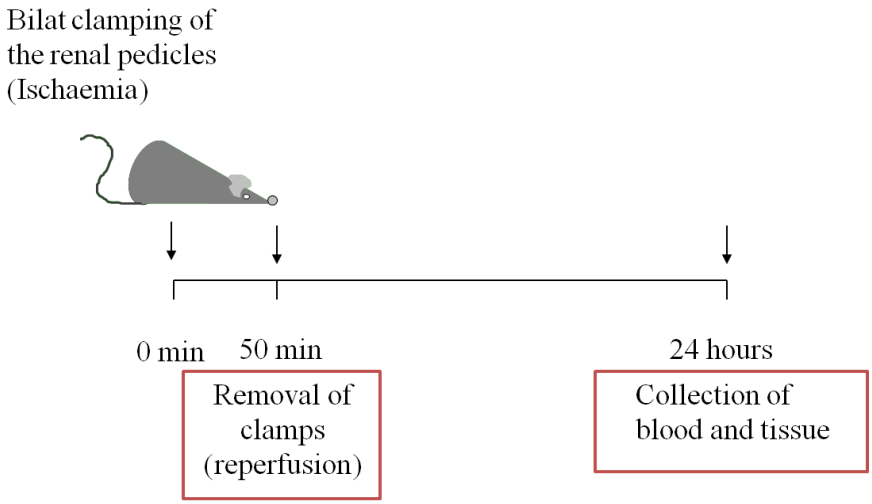


Figure 4. Summary of the IR protocol used for induction of native kidney IRI

2.4 Harvesting blood and tissue samples

2.4.1 Collection of blood and extraction of serum

24 hours after the initiation of reperfusion injury blood samples were collected in 1.5 ml eppendorf tubes following cardiac puncture. On average 300-400 μl of blood was collected by this method for functional analysis. Blood samples were kept in room temperature for 30 minutes to coagulate and then centrifuged for 15 minutes at 13,000 rpm. Separated serum samples were transferred to fresh tubes and centrifuged again at 13,000 rpm for 5 minutes to remove potential contaminating red blood cells. The serum samples were then stored at -20°C or -80°C before being used for measurement of renal function.

2.4.2 Collection of tissue

Following cardiac puncture and removal of blood sample, kidneys from both sides were removed surgically and cut into two through a coronal plane. One half of the kidney was placed in 4% formaldehyde (Sigma-Aldrich) for fixation and was later embedded in paraffin wax. Three quarters of the other half was immersed in OCT (optimal cutting temperature) compound (Raymond A. Lamb, London, UK) that was added to an aluminum mould suitable for mounting in a cryostat and was snap frozen in liquid nitrogen for immunohistochemistry studies. The remaining quarter was placed in a cryovial tube, snap frozen and kept in -80°C for potential future RNA analysis.

2.5 Assessment of renal function

2.5.1 Measurement of Blood Urea Nitrogen

Blood Urea Nitrogen (BUN) is the measurement of urea in the blood which is a by-product of protein that is metabolized in the liver and removed from the circulation by the kidneys. The level of blood urea reflects renal function in that when there is impairment in renal function, this level increases as the kidney cannot effectively filter and dispose of the urea.

BUN was measured using a diagnostic kit (BUN Infinity) from Sigma-Aldrich. The functional basis of this kit is degradation of urea by urease enzyme which forms ammonia. The ammonia then binds to 2-oxoglutarate to make glutamate. Glutamate dehydrogenase catalyses this reaction which results in oxidation of NADH (nicotinamide adenine dinucleotide) and formation of NAD. NADH oxidation leads to a reduction in absorbance at 340 nm optical density (OD), proportional to the initial concentration of urea.

To measure the BUN using the above kit, 5 µl of serum was added to 500 µl of BUN assay reagent. The mixture was inverted 5-6 times to allow equilibration. The absorbance was then read at 340 nm at time 0 and at 60 second. BUN level was calculated by comparing this change of absorbance in 60 seconds with a reference standard.

Dr Zhou in our laboratory had previously shown an excellent correlation between BUN and creatinine levels in IRI setting shown in Figure 5. For this reason BUN levels were used as an indicator of renal function in all IR experiments.

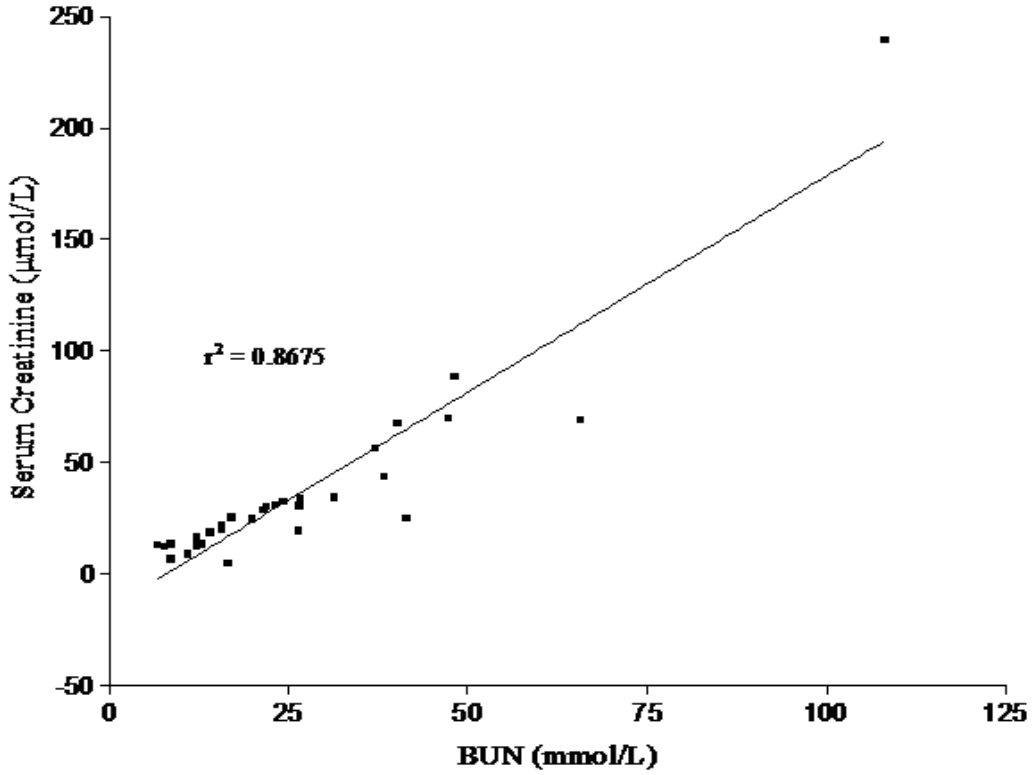


Figure 5. Correlation between creatinine and BUN measurement in mouse serum. Data generated by Dr Wuding Zhou.

2.6 Paraffin fixing and periodic acid schiff (PAS) staining

Kidney tissues fixed in 4% formaldehyde were embedded in wax and using a sledge microtome, were cut at 2 µm thick sections. The sections were then floated in a 40°C water bath and collected on a glass slide and left on the bench at room temperature to dry. The slides were then put in 60°C oven for a minimum of 1 hour for the wax to melt. The slides were then transferred to xylene to wash away the wax. Afterwards they were hydrated by passing through 100%, 90% and 70% ethanol solutions (40 seconds in each). They were then put in 1% periodic acid for 10 minutes following which they were washed in running water for 5 minutes. They were covered with Schiff's reagent for another 10 minutes and washed for 5 minutes. Slides were then put in haematoxylin solution for 5 minutes and then washed for 10 minutes and dehydrated by passing

through 70%, 90% and 100% ethanol solutions. They were dipped into xylene for 5 minutes and mounted in DPX (Distrene, Plasticiser, Xylene) mounting medium. After drying for a few hours they were reviewed using a light microscope.

2.7 Immunohistochemistry

4 µm frozen tissue sections were cut from snap frozen tissues for immunohistochemistry studies using a cryostat microtome (Bright, UK). Sections were transferred to glass slides and left in room temperature for 30 minutes to dry. If the slides were not used for staining on the day, they were stored in -80°C for future analysis.

2.7.1 Immunohistochemical staining for C3d

For FITC-labeled secondary antibody staining, the slides were initially fixed in acetone for 10 minutes and air-dried (if they were removed from -80°C freezer, they were left to dry for 30 minutes before staining). Slides were then immersed in PBS for 5 minutes and subsequently in 20% serum (of the species from which the secondary antibody was raised in, in this case goat serum) in PBS for 1 hour to block unspecific antibody binding. Slides were briefly washed with PBS and incubated with primary antibody for detection of C3d for 1 hour in a humidified chamber in room temperature. This was a purified immunoglobulin fraction of rabbit anti-human serum which is cross-reactive with mouse C3d (Mariathasan et al., 2006). After three washes in PBS for 5 minutes each, slides were incubated for 1 hour with FITC-conjugated goat anti-rabbit IgG in the humidified chamber at room temperature. Following three 5 minute washes with PBS and one wash with water to remove any unbound secondary antibody, slides were mounted using fluoromount and viewed with an immunofluorescence microscope.

For MASP-2 staining, rat anti-mouse primary antibody, kindly donated by Professor Schwaeble's lab was used followed by goat anti-rat FITC antibody for detection (Schwaeble et al., 2011).

2.8 Mouse kidney transplant

An isograft mouse model of kidney transplantation was used for this project. Dr Farrar who provides microsurgical services at our centre and is skilled in mouse kidney transplantation performed the surgery for identification of the role of complement in IRI. Dr Qijun Li, a research fellow in our department and experienced in microsurgical techniques, performed the surgical procedures for assessment of the therapeutic effects of anti-MASP-2 antibody. Donor and recipient mice were on C57BL/6 (B6) background to eliminate the risk of immune mediated rejection. Female mice were used for all transplant experiments. Mice were on average 8 to 10 weeks of age and weighed about 19-23 grams. All of these surgical procedures were performed on a warm mat and animals were anaesthetized with inhaled isoflurane just before start of the procedure. An established protocol in the unit was used for transplant experiments which was extracted from previous published methods by Kalina and Mottram (Kalina and Mottram, 1993) and later by Zhang and colleagues (Zhang et al., 1995). The left kidney was surgically removed from the donor with the renal vein and a renal arterial patch. After separating the donor ureter from the surrounding connective tissue, it was cut close to the bladder. The donor kidney was then put on ice for 30 minutes (cold ischaemia time) while the recipient was prepared. The right native kidney was surgically removed from the anaesthetized recipient and the aorta and the inferior vena cava (IVC) of the recipient were closed off using microaneurysm clamps. Small incisions were made in the aorta and IVC of the recipient for anastomosis of the respective vessels from the donor

kidney which was placed in the left iliac fossa. An end to side anastomosis of the donor aortic patch to the recipient aorta and of the donor renal vein to the recipient IVC was performed after which the microaneurysm clamps were removed and the kidneys were observed to ensure the transplanted kidney was appropriately reperfused (turning pink following de-clamping of the vessels). Implantation surgery in general took 40 minutes which is considered warm ischaemia time. To connect the ureter, two small incisions were made in the recipient bladder (one entrance and one exit). The donor ureter was pulled through both incisions following which 2 sutures were made between the entry incision of the recipient bladder and the connective tissue around the donor ureter to create some stability. The excess part of the donor ureter was removed at the exit incision site and the site was then closed with three sutures. 0.5 ml of warm saline was put in the abdominal cavity and the abdominal incision was sutured. The animal was then kept in 28°C chamber for 24 hours to recover. On day 5 the second native kidney was removed under anesthetic (isoflurane), and on day 6 the animal was sacrificed. Blood samples were taken at this time following cardiac puncture for measurement of BUN as an indicator of renal function. The transplanted kidney was removed and with a coronal cut was split in half. One half of the kidney was fixed in 4% formaldehyde for future paraffin staining. The other half was divided in 2 parts (one quarter and three quarters). The larger piece was snap frozen in liquid nitrogen for immunohistochemistry staining and the other piece was kept in liquid nitrogen for RNA extraction and measurement of inflammatory marker gene expression if required.

2.9 Therapeutic use of anti-MASP-2 antibody in mouse kidney transplantation

In collaboration with Professor Schwaeble and Omeros Corporation Company (Seattle) that had made an effective anti-MASP-2 antibody (AbyD 04211), kidney transplants

were performed using the antibody and the provided isotype control antibody based on this protocol:

Anti-MASP-2 monoclonal IgG2a antibody and its isotype control were supplied frozen in clear vials of stock solution. The antibodies were thawed and aliquoted upon arrival and kept in -20°C freezer until planned to use to prevent frequent freeze-thaw cycles. The day prior to transplantation, the recipient mice were randomly injected (intra-peritoneal) with 0.6mg/kg of the anti-MASP-2 antibody or the isotype control, as recommended by the manufacturer on the basis of their bioavailability experiments. One dose (0.6mg/kg intraperitoneal) was considered sufficient to prevent MASP-2 activation for at least 7 days. The day after administration of the antibodies, transplants were performed using the above protocol. The only difference in these experiments compared to the transplant experiments in gene knockout mice was that the mice were not scarified at day 6. Small amount of blood (40-50 µl) was obtained through a tail incision on day 6 and the animal was sacrificed at day 7. Dr Qijun Li, a research fellow in our lab who had great experience in mouse microsurgery, performed these transplantations.

2.10 Statistical analysis

Unpaired t-test was used for the statistical analysis of all paired data sets with the use of GraphPad prism software (version 5).

3 Examination of the role of C3a in renal IRI

3.1 Hypothesis and aims of the study

As discussed above, complement activation is known to have a role in IRI. An important obstacle in translating basic laboratory results into clinical practice has been the lack of understanding of the specific action of complement. Complement generates

several effector products such as C3a, C5a and C5b-9 that interact with receptors on cells or insert directly into cell membrane causing injury to the target tissues (Sim and Tsiftsoglou, 2004). While some studies have defined the terminal attack complex (C5b-9) as a key mediator of IRI in the kidney (Zhou et al., 2000), others have identified the small cleavage fragments known as C3a (Ducruet et al., 2008) and C5a (de Vries et al., 2003a) to be of significant importance in inducing injury following cerebral and kidney IR respectively. Indeed, animal studies using C5a inhibitors (antibody or antagonist) have shown the potential for preventing reperfusion damage in organs such as the heart, hind-limb and the kidney (Arumugam et al., 2003, Amsterdam et al., 1995, Woodruff et al., 2004). There is little information available for the role of C3a and the intracellular pathway following activation of its receptor on the kidney tubular epithelial cell, and this seems important since C3a can have functions that are distinct or overlapping compared to C5a. I hypothesized that absence of C3aR would have a protective effect in native kidney IRI.

My aim was to compare the injury induced by IR in native WT mouse kidney vs C3aR-/- kidney, to investigate if the C3aR-/- strain are protected from injury. The ultimate plan was to identify the relative importance of C3a in the mechanism of renal IRI and inform strategies to prevent receptor activation if C3aR turned out to be a significant mediator of the injury.

3.2 Experimental design

To investigate the involvement of C3a in kidney IRI, C3aR-/- mice were genotyped to make sure they lack expression of the receptor gene. Then using an established model to induce IRI, C3aR-/- and WT mice were subjected to 50 minutes of renal ischaemia (by bi-lateral clamping of the native renal artery and vein) followed by 24 hours of

reperfusion (after removal of the clamps). A pilot study including eight mice in each group was initially performed and the numbers in each group were subsequently increased to enable more definitive comparison of the two groups. The serum and kidney tissue were harvested 24 hours after reperfusion. The intention was to look at the difference in blood urea nitrogen and tissue injury (on kidney histology samples) between the 2 groups. The plan was to investigate the intracellular mechanism of C3aR activation leading to tissue damage if the WT mice had more injury compared to the C3aR-/- strain.

4 Results

4.1 Characterization of C3aR-/- mice by genotyping

It was essential to confirm the phenotype of the C3aR-/- mice prior to induction of IRI to ensure the mice used in the experiment have no C3aR. To achieve this, genotyping of the mice was performed using their tail as a source of DNA.

Mouse tails from C3aR-/- and WT mice were used to produce genomic DNA as described in section 2.6.1. Using the appropriate primers for C3aR and GAPDH, the genes were amplified and semi-quantitative PCR product was determined for each sample using gel electrophoresis.

Figure 6 shows an agarose gel stained with ethidium bromide and examined under UV light, following C3aR and GAPDH gene amplification using semi-quantitative PCR from WT and C3aR-/- mouse. A positive 230 base pair band for C3aR is visible in the WT mouse but is absent in the C3aR-/- mouse. The result of this experiment confirms the C3aR deficient genotype, enabling mice with this result to be used in future experiments.

The figure shown here is a representative of C3aR genotyping. About 20 mice were genotyped and the homozygotes and heterozygotes were separated and allowed to breed. The offspring of the homozygotes were used in experiments. The progeny of the heterozygotes were genotyped for detection of homozygotes as above.

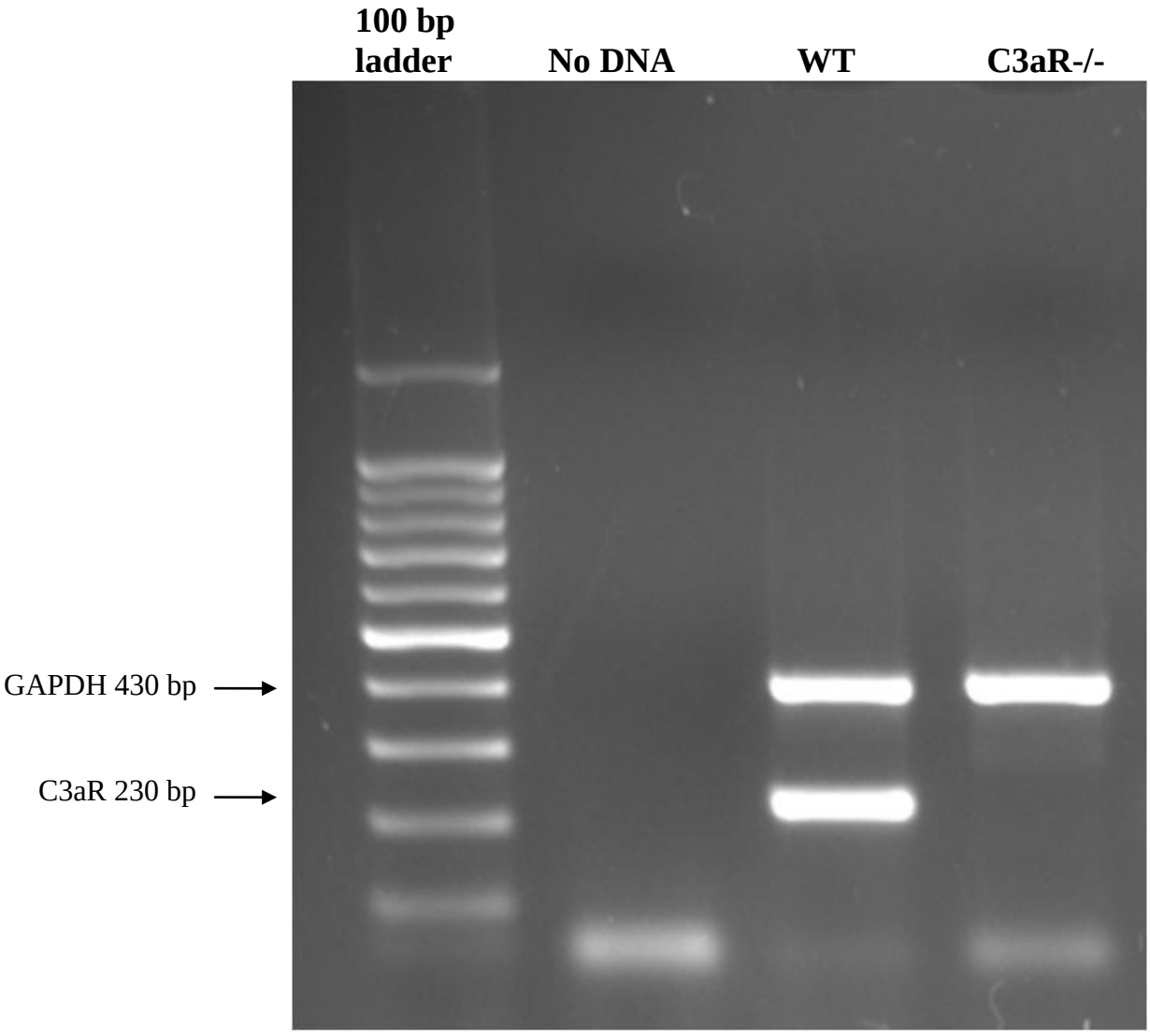


Figure 6. Detection of C3aR in WT and C3aR-/- mice using PCR. Genomic DNA was prepared from WT and C3aR-/- mouse tails. Comparison was made between semi-quantitative PCR products from amplification of C3aR gene and GAPDH as control. In each PCR reaction 10 ng of DNA was used.

4.2 IRI in WT and C3aR-/- mice

The native kidney IR protocol described in section 2.7 was used in this experiment where renal pedicles on both sides were clamped for 50 minutes. 24 hours after clamp removal, animals were sacrificed and blood sample was taken for BUN measurement and both kidneys were harvested for histology experiments.

The pilot experiment consisted of 8 WT and 8 C3aR-/- mice, both on C57BL/6 background. The average BUN measurements in both groups are shown in Figure 7.

The average BUN for WT non-injured mice was 6 mmol/l.

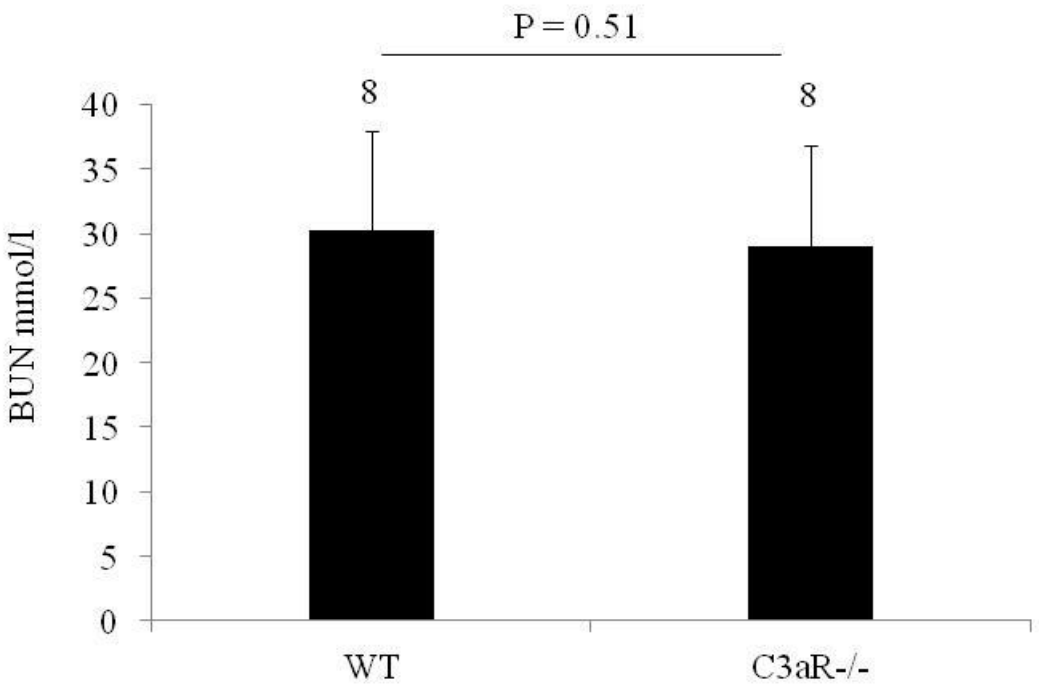


Figure 7. Effect of renal IRI on renal function in WT and C3aR-/- mice. Mice in each group underwent 50 minutes of bilateral renal ischaemia. Blood samples were obtained 24 hours after removal of clamps and used for BUN measurement. Numbers above each column represent the number of animals in each study. The values are means +/- SD (standard deviation). P value <0.05 is considered significant.

Following the pilot experiment, I had become experienced in inducing IRI with consistent results. Although there was no significant difference in renal function

between the 2 groups, the C3aR-/- mice had slightly better renal function. I therefore repeated the experiment using 8 WT and 11 C3aR-/- mice (which were available at the right age and size), to confirm whether or not a significant difference could be detected with an enlarged number of mice. The result of this second experiment comparing the renal function between WT and C3aR-/- mice after 50 minutes of ischaemia and 24 hours of reperfusion is shown in Figure 8.

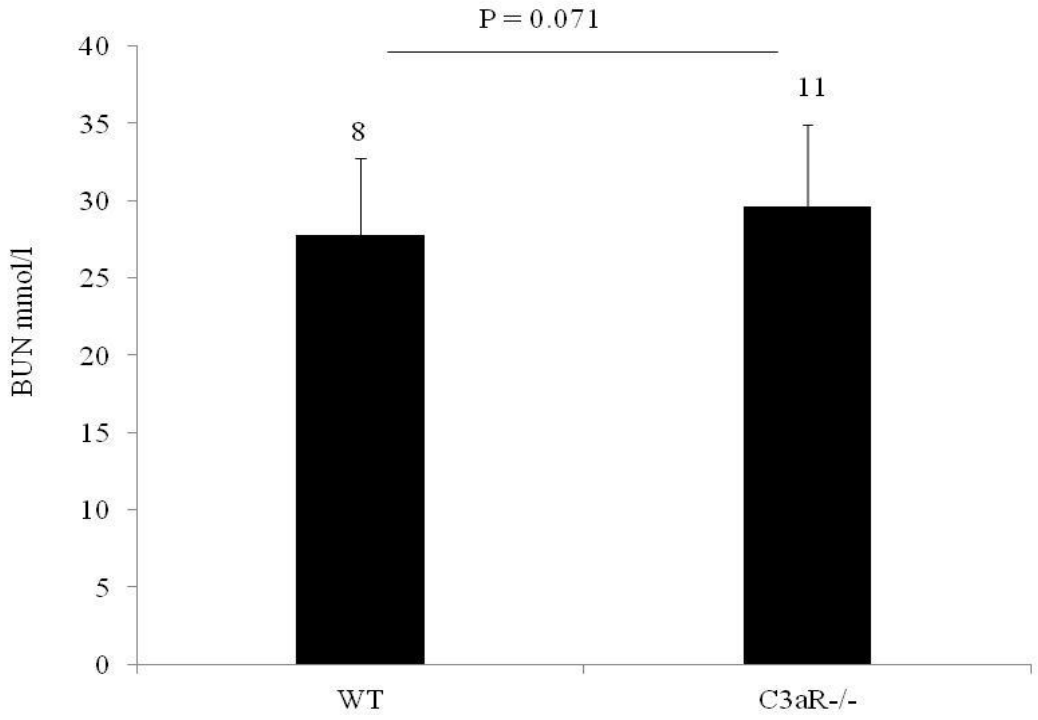


Figure 8. Effect of renal IRI on renal function in WT and C3aR-/- mice (2). Mice in each group underwent 50 minutes of bilateral renal ischaemia. Blood samples were obtained 24 hours after removal of clamps and used for BUN measurement. Numbers above each column represent the number of animals in each study. The values are means +/- SD (standard deviation). P value <0.05 is considered significant.

As shown in Figure 8 the repeated experiment did not confirm my hypothesis that absence of C3aR can protect the mice from IRI. In fact this time the C3aR-/- mice had slightly worse renal function compared to the WT although this was not statistically

significant. Both experiments were performed in similar conditions and there was a short interval between them. I pooled the result of both experiments and compared the values between the 2 groups, the result of which is shown in Figure 9.

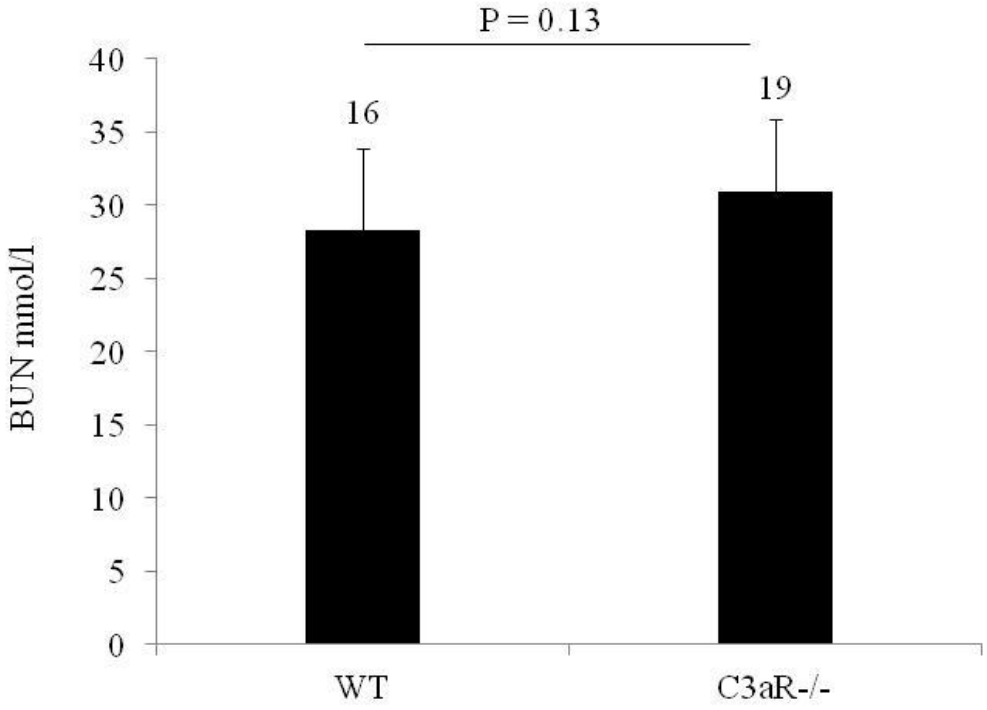


Figure 9. Effect of renal IRI on renal function in WT and C3aR-/- mice (3). Pooled result from the 2 experiments measuring BUN as a marker of renal function following 50 minutes of bilateral renal ischaemia and 24 hours of reperfusion. Numbers above each column represent the number of animals in each study. The values are means +/- SD (standard deviation). P value <0.05 is considered significant.

In addition to the BUN measurement in WT and C3aR-/- mice, the kidneys were examined microscopically for tissue damage as described below. Figure 10 shows the morphology of normal non-injured kidney tissue in which the normal tubular and basement membrane structure are highlighted.

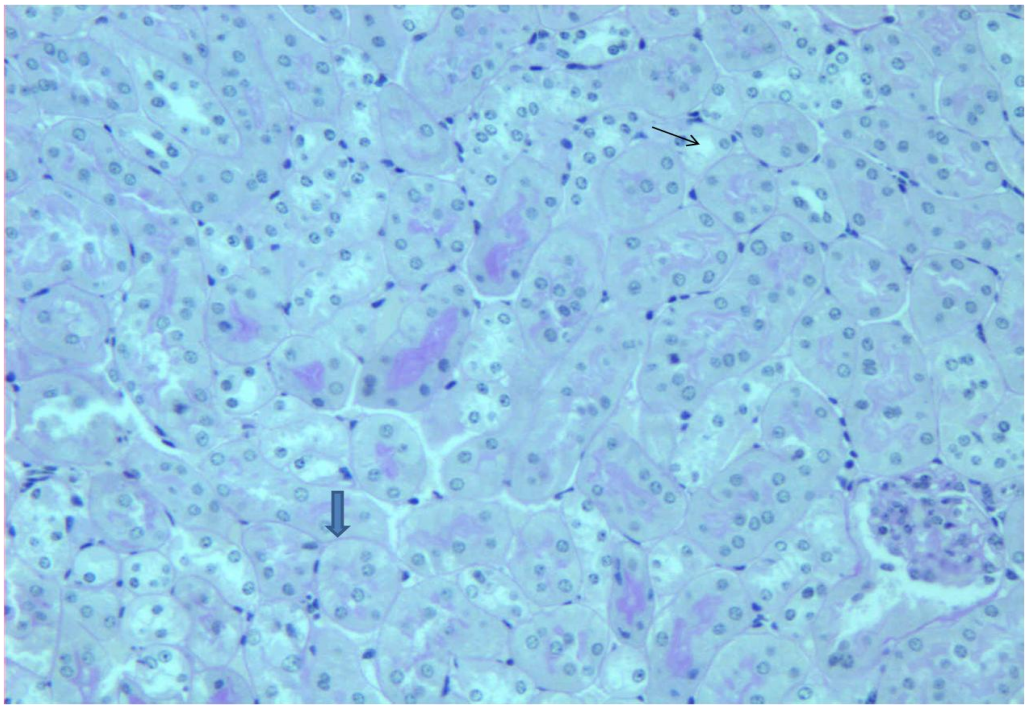


Figure 10. Histology of non-ischaemic kidney tissue. Normal non-ischaemic kidney tissue from WT B6 mouse, stained with periodic acid Schiff. The thin arrow points to a tubular lumen and the thick arrow points to an intact basement membrane (magnification x 40).

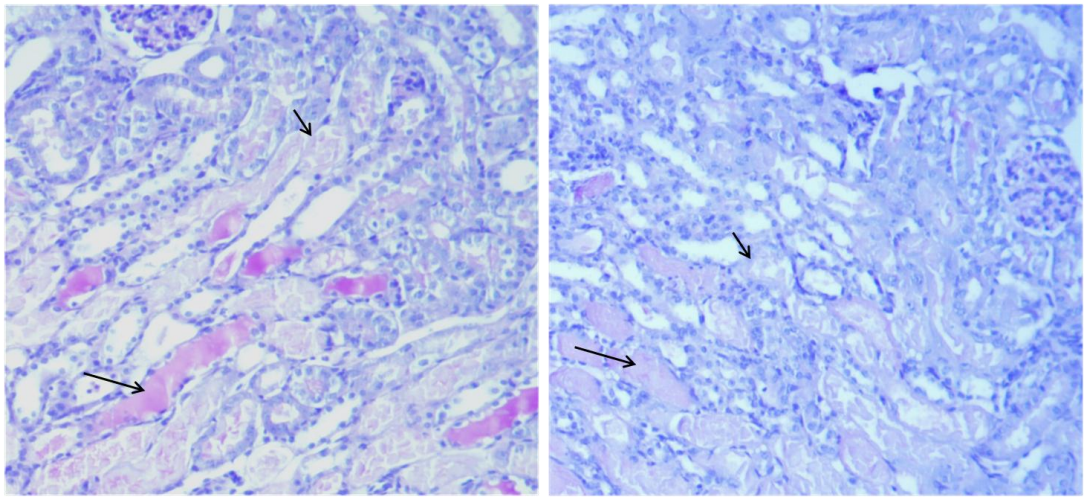
The paraffin embedded kidney tissues from the WT and C3aR-/- mice were prepared and cut followed by PAS staining as described in section 2.10. Four coronal sections were processed from each animal in the experiment and in a blinded fashion examined using a light microscope. Tubular damage was assessed using the 5 point scale described below (Table 5).

Percentage of injury on histology	Score
0% (Normal kidney)	0
<10%	1
10-25%	2
25-50%	3
50-75%	4
>75%	5

Table 5. Scoring system used for assessing kidney tubular damage

For assessing tubular damage in histology sections, the above well-established scoring system (Kummer et al., 2007) was used in which 0 stands for no injury, 1 is when there is <10% tubular damage, 2, 10 to 25% damage, 3, 25 to 50% damage, 4, 50 to 75% damage and 5, more than 75% damage. The score was given based on the review of the four coronal sections for each animal.

Figure 11 demonstrates the tissue morphology of the WT and C3aR-/- kidney following IRI. The images are representative of each group (all kidney samples from both groups were processed and analyzed). As shown in the pictures, in both samples there is significant tissue damage such as thinning of the tubular epithelium, tubular dilatation, patchy loss of some tubular cells and tubular casts. The histological damage was proportional to the functional impairment of the kidneys.



WT C3aR-/-

Figure 11. Effect induced by ischaemia-reperfusion on renal morphology. PAS staining of WT and C3aR-/- mouse kidney following 50 minutes of ischaemia and 24 hours of reperfusion. The small arrows show areas of tubular damage and the longer arrows indicate tubular casts (magnification x 25).

Comparing the histology samples using the 5 point scale described above, there was no significant difference between the two groups regarding tubular damage (P=0.44) which was expected from the functional data. Tubular damage scoring of the WT and C3aR-/- kidneys are shown in Table 6.

Mouse group	Mean severity score ± SD
WT	3.06 ± 0.85
C3aR-/-	3.26 ± 0.73

Table 6. Tubular injury in WT and C3aR-/- mice. SD: standard deviation.

4.3 Discussion

Various elements of the complement pathway have been shown to have a role in induction of IRI in different organs. Anaphylatoxins C3a and C5a, which are by-products of complement activation, are known to be mediators of many inflammatory reactions (Kohl, 2001). C5a has been shown to mediate kidney IRI (de Vries et al., 2003a), and inhibition of C5a has been demonstrated to have a protective role (Arumugam et al., 2003). Although C3a has been shown to induce the production of chemokines in kidney tubular epithelial cells in response to hypoxia (Thurman et al., 2007), the direct effect of C3a in kidney IRI has not been determined.

To assess the role of C3a in kidney IRI, I used C3aR-/- mice in comparison with WT mice in a native model of kidney IRI in which 50 minutes of ischaemia was induced by clamping the renal vessels followed by 24 hours of reperfusion. Contrary to my expectation, absence of C3aR did not protect the mice from kidney IRI and mice with C3aR deficiency had similar functional and histological damage in their kidney following the IR. This is suggestive that in this model, C3a-induced inflammation is not the main cause of kidney damage after IRI and other factors are more effective in inducing the observed damage.

There are some factors that might have influenced the outcome of this study. In most kidney IRI studies, the ischaemia time is significantly shorter (24-30 min) than the 50 minute time point I have used. 50 minute ischaemia was established in our lab by Dr Zhou and was routinely used in all IRI studies. It is possible that in my hands, 50 minutes of injury was too much to allow differences between the two groups related to complement activation be demonstrated. It is plausible that long periods of ischaemia result in complement activation initially which then lead to tissue necrosis if the injury is not eliminated.

The strain of mice is another important factor in complement studies. For example, it has been shown that different strains of mice have different capacity to produce C5a (Patel et al., 2008). However, the fact that WT and C3aR-/- animals in this experiment were on the same background should eliminate this potential effect.

In any event, the results suggest that it would be unlikely that therapeutic C3a inhibition or blockade have a significant effect on prevention of IRI in the experimental model described here.

Considering the importance of complement activation in IRI and the reported detrimental actions of C5a and C5b-9 in native and transplanted kidneys, I went on to investigate which complement activation pathway could be the trigger for inducing injury in kidney transplantation. Availability of mice deficient in particular elements of complement which would allow investigation of each specific pathway allowed me to design an informative set of experiments. I explain this in more detail in the next chapter.

5 Investigating the importance of complement activation pathways in a mouse kidney isograft model of IRI

The focus of this part of the project was to assess the participation of each of the complement pathways in inducing reperfusion injury in a mouse renal isograft model. A transplant model of IRI was chosen for these experiments as there are several aspects that are different from native kidney IRI. For example, in transplantation the organ is denervated and handled and undergoes both cold and warm ischaemia compared to only warm ischaemia in the native model. These differences are likely to affect how the

complement is activated and the pathway which is most significant in inducing tissue damage.

Previous work from our laboratory indicated that C4-deficiency, unexpectedly, does not have a protective role in native renal IRI (Zhou et al). This is surprising as MBL has been shown to play a role in a similar model (Moller-Kristensen et al., 2005, de Vries et al., 2004). The question which arises based on these findings is that if MBL is important but not C4, then how this could be explained, since C4 is an obligatory intermediate in the LP according to the text book description. This could suggest that MBL is able to result in cleavage of C3 independently of C4, or MBL could contribute to another, as yet undefined mechanism leading to reperfusion injury. Either way, considering the fact that MASP-2, an essential serine protease of the LP interposed between MBL and C4, is required to convert the pattern recognition function of MBL into a downstream effector function, I proposed that if MASP-2 is required for reperfusion injury and is shown to lead to C3 cleavage independently of C4, this would suggest the possibility of a novel C4-bypass pathway resulting in the cleavage of C3. I therefore tested the role of MASP-2 in the induction of renal isograft reperfusion injury and determined if it has an action that is independent of C4. The role of the AP of complement activation is also of interest, since the AP can amplify the cleavage of C3 into C3b initiated by the LP or CP. I aimed to assess the potential effect of the AP in the kidney transplant model of IRI using factor B deficient (FB-/-) mice.

Previous studies have demonstrated that the kidney (in both human and mice) is able to produce several complement components including C4 and FB (Welch et al., 1996, Passwell et al., 1988, Welch et al., 1993). For this reason, in transplant experiments using C4-/- and FB-/- mice, the donor kidney was taken from the C4-/- and FB-/- mice respectively where the recipient was deficient in the same component as the donor

organ. MASP-2 enzyme however, has not been shown to be produced outside of liver (Stover et al., 1999a), therefore in experiments assessing the role of MASP-2 deficiency in IRI, WT donors were used in MASP-2 deficient recipients with the expectation that the donor kidney would not be able to produce MASP-2 to affect the experiment result.

5.1 Experiment design

I designed the initial experiments focusing on the role of MASP-2 in the LP and on the role of C4 in the LP and CP, followed by the role of FB in the AP. The donor-recipient groups were arranged as shown in Figure 12 with 8 mice transplanted in most groups. WT to WT transplants were proposed as the control group and transplantation of WT mouse kidney to the MASP-2 deficient mouse was planned to assess the role of MASP-2 enzyme following IRI. As MASP-2 is reported to be only produced in the liver, donor kidney from the WT mouse was used in these experiments (validated by staining of MASP-2 to assess the presence of the enzyme in the liver and transplanted WT kidney, Figure 15). For investigating the role of C4, C4-/- mice were used as both donor and recipient as kidney is known to be a source of C4 (discussed above). To determine if absence of C4 provides any additional benefit to MASP-2 deficiency in preventing IRI an additional transplant group was performed consisting of transplants from C4-/- donors to recipients with combined deficiency of C4 and MASP-2. The idea was that if C4 has a protective role in IRI other than its effect on the LP activation, the C4-/-/MASP-2-/- mice would have less injury compared to the mice with MASP-2 deficiency alone.

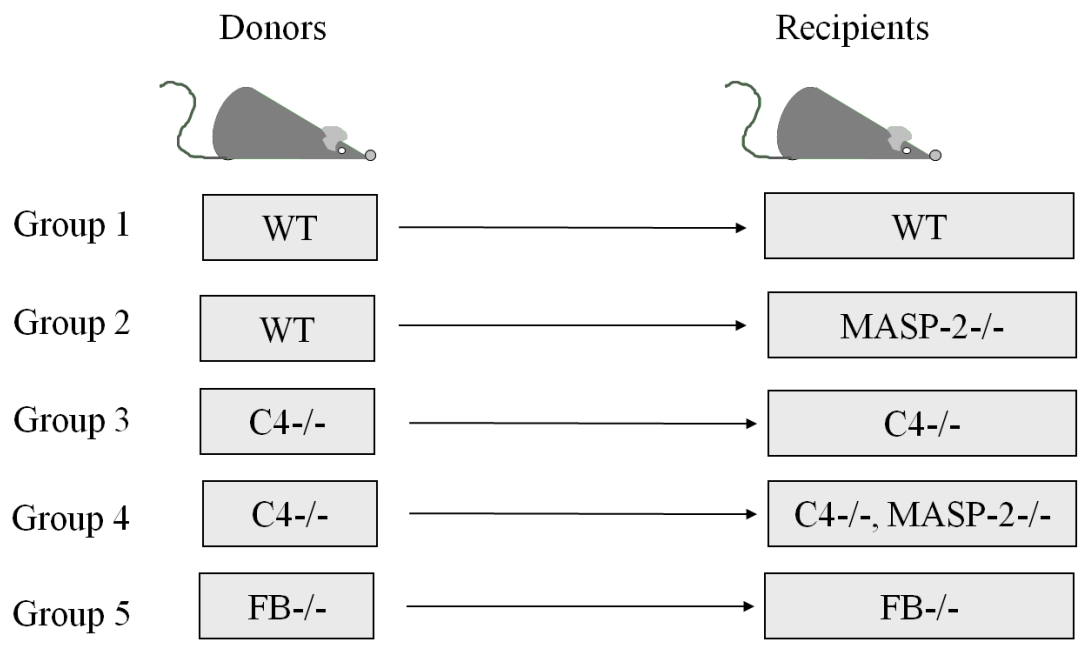


Figure 12. The experimental groups designed for assessment of the role of the LP, the CP and the AP in a transplant model of renal IRI. Transplant between WT animals (group 1) was performed as control. In the second group, WT kidneys were transplanted into MASP-2-/- animals to assess the role of the LP activation in transplant kidney IRI. In the 3rd group, kidney from C4-/- donor was transplanted to C4-/- recipient to assess the role of both the CP and the LP as both pathways are dependent on C4. Transplant in the fourth group was performed to investigate if absence of C4 has any additional benefit to deficiency of MASP-2. In this group, donor kidney was a C4-/- mice, and the recipient was deficient in both MASP-2 and C4, due to the fact that the C4-/- donor kidney would not be able to produce MASP-2 and essentially be deficient in both C4 and MASP-2. In the fifth group both the donor and recipient mice were FB-/- to allow assessment of the role of the AP in this model of kidney IRI.

Transplants were performed as described in section 2.12. Figure 13 shows a summary of the procedure. After 30 minutes of cold and 40 minutes of warm ischaemia, the donor kidney was transplanted to the recipient, and then the left recipient kidney was immediately removed. The second recipient kidney was removed on the 5th day post transplantation and the mice were sacrificed a day after at which time their blood and kidney tissue were collected for analysis. This procedure was adopted after a number of control studies in our department had shown that bilateral removal of recipient kidneys at the time of transplantation leads to high death rates of the animals. Also earlier (than 5 days) removal of the second native kidney from the recipient was associated with high

mortality. The current protocol generated the most consistent results with the least number of deaths in the transplanted animals.

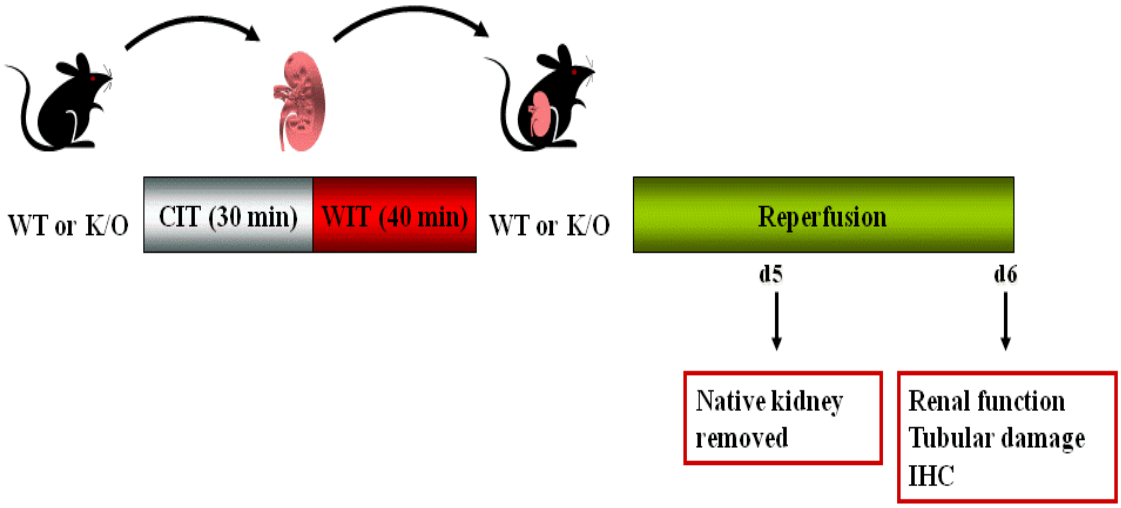


Figure 13. Summary of the mouse kidney transplantation protocol used in the experiments. Kidney from the donor mouse was removed and was kept on ice for 30 min (CIT, cold ischaemia time) in which time the recipient was prepared for transplantation. The right recipient kidney was removed and the donor kidney was transplanted within 40 minutes (WIT, warm ischaemia time). The native left recipient kidney was removed on the 5th day after transplantation and the animal was sacrificed on day 6 when blood and tissue samples were collected for assessment.

5.2 Results

Figure 14 shows the recipient BUN results from the first four of the experimental groups described above. Eight mice were transplanted in each group and one mouse from each group died prior to the end of the experiment. The experiments were performed over a period of 3 months using the same room and equipment to reduce the variability between groups to minimum.

5.2.1 Effect of MASP-2 and C4 on IRI in mouse kidney isografts

Transplants were made in the above 4 groups to assess the role of the lectin and the classical pathway in the transplant model of renal IRI. As demonstrated in Figure 14,

the WT recipients had significant amount of kidney injury at day 6 with an average BUN of 38 mmol/l. In contrast, the MASP-2-/- recipients were protected with an average BUN of 15 mmol/l (P value= 0.015). Considering the fact that absence of MASP-2 completely abrogates LP activation, the renal protection in MASP-2-/- animals is suggestive that the LP does play a role in inducing IRI. However, absence of C4 did not protect the kidneys from transplant IRI, since renal function in C4-/- mice was similar to the WT group with an average BUN of 42 mmol/l. This is suggestive that the CP, which is dependent on C4 for its activation, does not play a role in this model of injury. As C4 is theoretically required for LP activation as well, and considering that absence of MASP-2 is protective and absence of C4 is not, this suggests that LP activation was independent of C4 in these experiments. To investigate if absence of C4 leads to any additional benefit to MASP-2 deficiency in protecting from IRI, transplants were performed from C4-/- donors to C4-/-/MASP-2-/- recipients. As evident in Figure 14, absence of C4 did not add to the protective effect of MASP-2 deficiency and the average BUN in this group was similar to the MASP-2 deficient only group at 17 mmol/l.

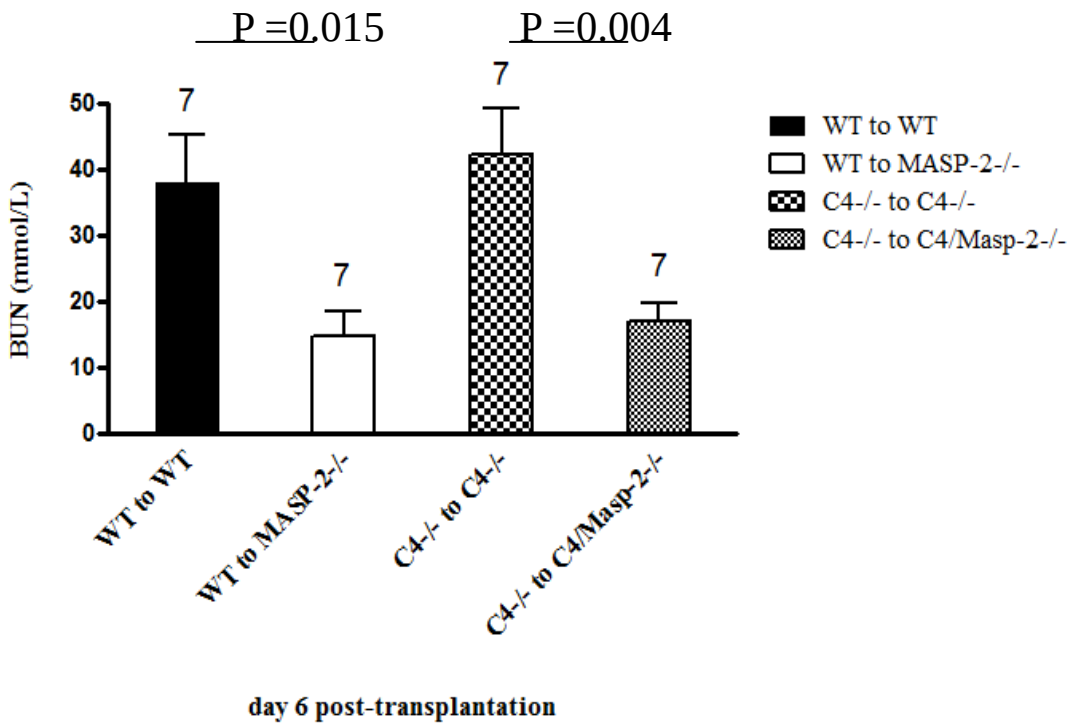


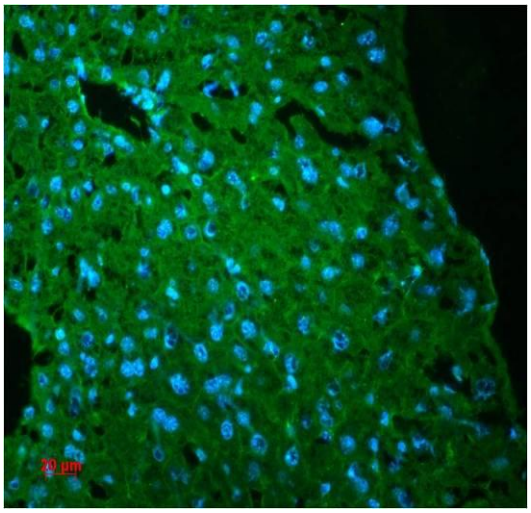
Figure 14. Absence of MASP-2 is protective in a transplant model of kidney IRI. Measurement of renal function in four groups of transplant recipients 6 days after transplantation. Transplantation was performed with 30 minutes of cold ischaemia and 40 minutes of warm ischaemia time with removal of one native kidney. The remaining native kidney was removed on day 5 and blood samples were collected a day after. The number above each column indicates the number of transplants performed in each group. Arrow bar on each column shows the standard deviation. P value of < 0.05 is considered significant.

These results indicate that the activation of MASP-2 enzyme is important in transplant kidney IRI and inhibition of this enzyme could potentially reduce the injury to transplanted kidneys. Interestingly, activation of the LP was independent of C4, suggesting that there is a bypass pathway available in which MASP-2 can cleave the C3 molecule without C4.

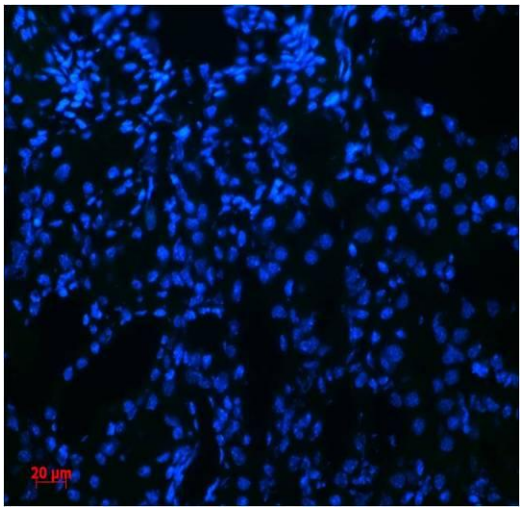
5.2.2 Expression of MASP-2 enzyme

To confirm that MASP-2 enzyme is not expressed in WT kidney as previously reported (and thus exclude an effect of MASP-2 in WT kidney transplanted into MASP-2-/- recipients), immunofluorecent staining of the enzyme was performed on frozen sections of 2 native kidneys from WT B6 mice and 2 WT kidneys transplanted into a WT

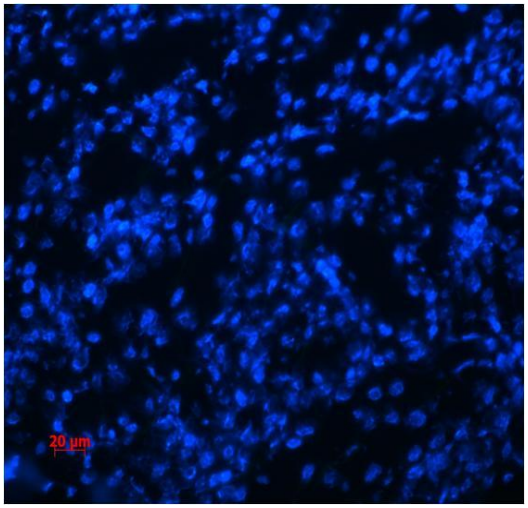
recipient (following ischaemic insult of the donor kidney). Frozen sections of the liver were also stained as positive controls, as it is well established that liver is the main source of MASP-2 production (Bornstein, 2008). There was no MASP-2 detected in either of the kidneys of the WT mice (native and transplanted). This suggests that it is unlikely that the WT donor kidney would have MASP-2 activation following transplantation in the MASP-2-/- mouse. Considering the significant reduction of injury in the MASP-2-/- mice, even if there was a supply of MASP-2 in the WT donor kidney, this could not have resulted in significant damage resulting from complement activation. Figure 15 shows the result of tissue staining for MASP-2 enzyme in the normal liver and kidney as well as in a transplanted kidney.



Liver



WT Kidney after transplantation



Non-ischaemic WT kidney

Figure 15. Immunohistochemical staining of MASP-2 enzyme in the liver, non-ischaemic and transplanted kidney of WT B6 mouse. The green fluorescent colour indicates the presence of the enzyme which is abundant in the liver and the blue colour is the Hoechst stain for cell nuclei. MASP-2 protein is not detected in the normal and transplanted WT kidney.

5.2.3 Tissue damage in the transplanted kidneys

To assess the kidney tissue damage following transplantation in each of the experiment groups and to find out if the kidney function measured by BUN had any correlation with kidney tissue damage, histological sections from the harvested kidneys of all

experiments (7 mice in each group) were stained with Periodic Acid Schiff and assessed by two examiners (myself and Dr Farrar) in a blinded fashion. The severity of tubular damage was assessed with the scoring system summarized in Table 5. As shown in Figure 16, WT kidneys transplanted into WT mice as well as C4-/- kidneys transplanted into C4-/- recipients had significantly more tubular damage compared to the MASP-2-/- recipients (both the MASP-2-/- that was the recipient of WT kidney and the C4-/-/MASP-2-/- mice that was the recipient of the C4-/- kidney). As shown in Table 7, the damage score was significantly higher for the WT and C4-/- recipients. The histology scores were in general proportional to kidney damage observed with BUN measurement (Figure 14) which was expected.

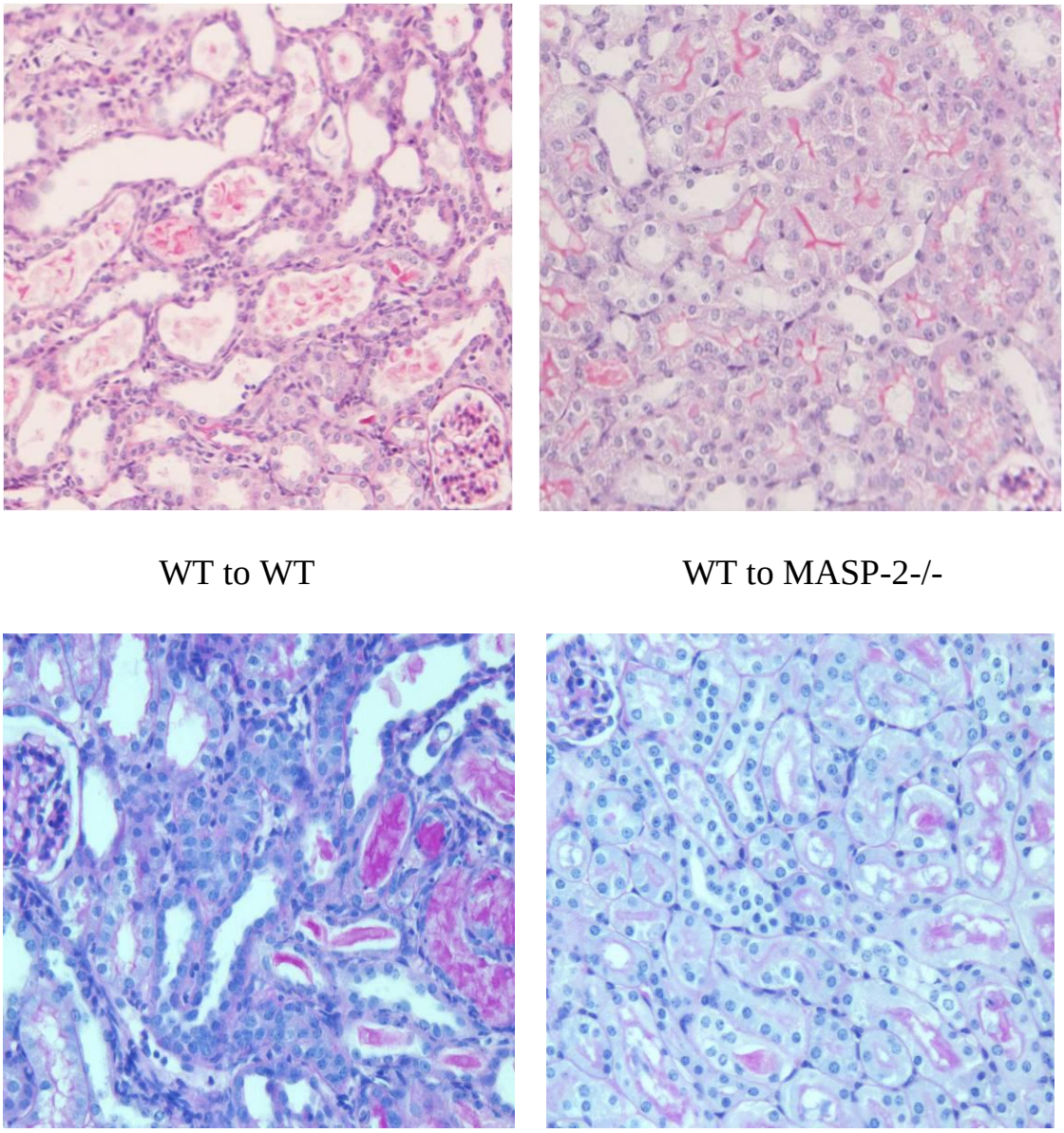


Figure 16. Effect of IRI on the renal tissue morphology in four transplant groups. Above are light microscopy images of representative histology samples from the kidney transplants in the four groups. In both WT and C4-/- transplant recipients, there are visible signs of tubular damage including tubular thinning and dilatation and formation of hyaline casts in the tubules, compared with the MASP-2-/- and C4/MASP-2-/- recipients where the kidney shows minimal signs of injury. PAS staining, x40 magnification

Isograft group (number of transplants)	Mean severity score ± SD
WT to WT (7)	3.14 ± 1.46
WT to MASP-/- (7)	1.57 ± 0.78
C4-/- to C4-/- (7)	3.29 ± 1.13
C4-/- to C4/MASP-/- (7)	1.71 ± 0.75

Table 7. Tubular damage score based on the histology of all transplants performed in the four experimental groups

The above table shows the average scoring ± SD (standard deviation) for tubular damage in the four transplant groups. The WT and C4-/- recipients had significantly more tubular damage in their kidneys compared to the MASP-2-/- and C4/MASP-2-/- animals. P value comparing the WT and MASP-2-/- recipient scores was 0.027 and between C4-/- and C4/MASP-2-/- recipient was 0.0215.

5.2.4 Immunohistochemical staining for complement deposition

In order to obtain evidence of complement activation in the injured tubules, I examined the staining pattern for C3d in all kidneys from the transplant experiments, using immunohistochemistry. C3d is a metabolite of C3b through iC3b, and its deposition on cells provides a measure of complement activation at that site. Staining the frozen sections of the kidney tissue from the transplant experiments, a large increase in C3d deposition was detected, notably at the basolateral surface of tubular epithelial cells in a circumferential pattern. The proximal renal tubules are the site of maximum tubular injury and correspondingly are the main target for C3 deposition after reperfusion of

ischaemic kidney. In the MASP-2 deficient mice, the tubular deposit of C3 was markedly reduced, consistent with reduced complement activation at the tubule surface in the absence of MASP-2. This suggests that activation of C3 on the basolateral surface of kidney tubules is dependent on MASP-2, and the lower C3d staining is either due to reduced C3 cleavage downstream of MASP-2 or due to reduced injury through a separate mechanism involving MASP-2. The C4-/- kidneys showed similar level of C3d staining as WT kidneys, which indicates that activation of complement at this site was independent of C4. C4-/-/MASP-2-/- recipients of the C4-/- kidneys had similar staining pattern to MASP-2-/- recipients emphasizing that activation of complement and hence C3d deposition had been reduced due to the absence of MASP-2. The extent and intensity of C3d deposition was assessed by 2 people (myself and Dr Farrar) in a blinded fashion.

The increase in intensity and extent of C3d deposition in WT and C4-/- recipients in the face of the worse renal function in these groups, could be an indication that activation of complement in the tubules at least to some extent is responsible for the observed tubular injury.

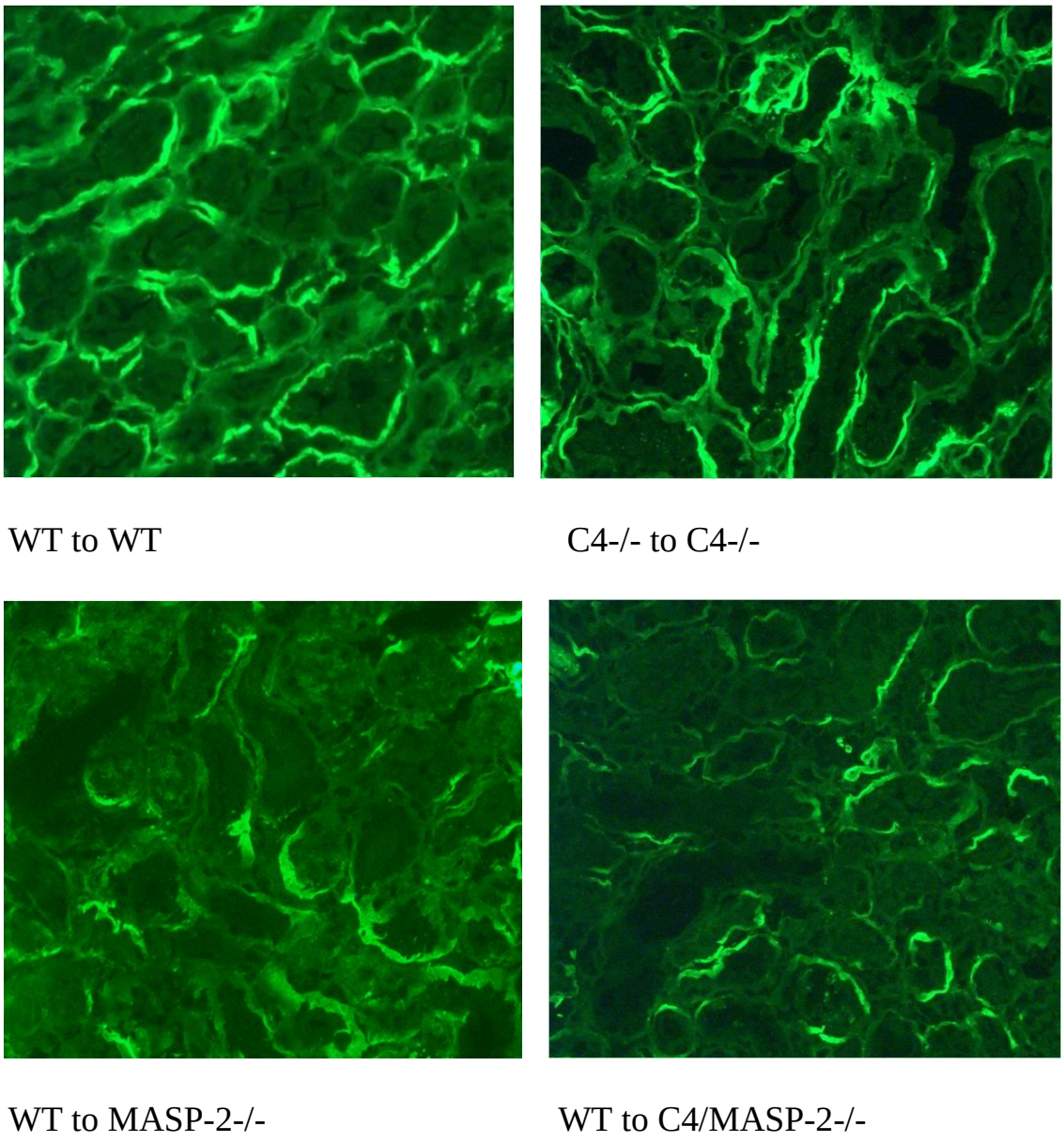


Figure 17. Immunohistochemical staining of the complement split product C3d in the four transplant groups. Frozen sections of the transplanted kidneys from the above four transplant groups were stained for C3d, using FITC labeled anti-C3d antibody. Deposition of C3d was observed in the basolateral surface of the tubular epithelial cells in all groups. However in WT and C4-/- recipients the deposition of C3d was more extensive and had more intensity compared with the MASP-2-/- and C4/MASP-2-/- recipients.

5.3 Role of the alternative pathway in transplant kidney IRI

To assess the role of the AP in the kidney transplant model of IRI, FB-/- animals were used. FB is an essential component required for activation of complement via the AP.

Although absence of FB and hence the AP activation in native kidney IRI has been shown to be protective of kidney tissue damage (Thurman et al., 2005), its contribution to tissue injury has not been investigated in a transplant IRI model. For this experiment, 2 transplant groups were designed with the aim of 6 transplants in each group: (1) WT to WT transplantation as the control group and (2) FB-/- to FB-/- transplants to assess the effect of the AP. As discussed earlier, due to the ability of WT kidney to produce FB, both donor and recipient were FB-/-. The average BUNs from the two groups on day 6 after transplantation are shown in Figure 18. In contrast to a report for a native kidney model (Thurman et al., 2003), absence of FB did not lead to significant protection form IRI, suggesting that activation of the AP does not play an important role in the kidney transplant IRI, either by triggering the complement cascade or by amplifying the effect of the LP (shown here to mediate renal transplant IRI).

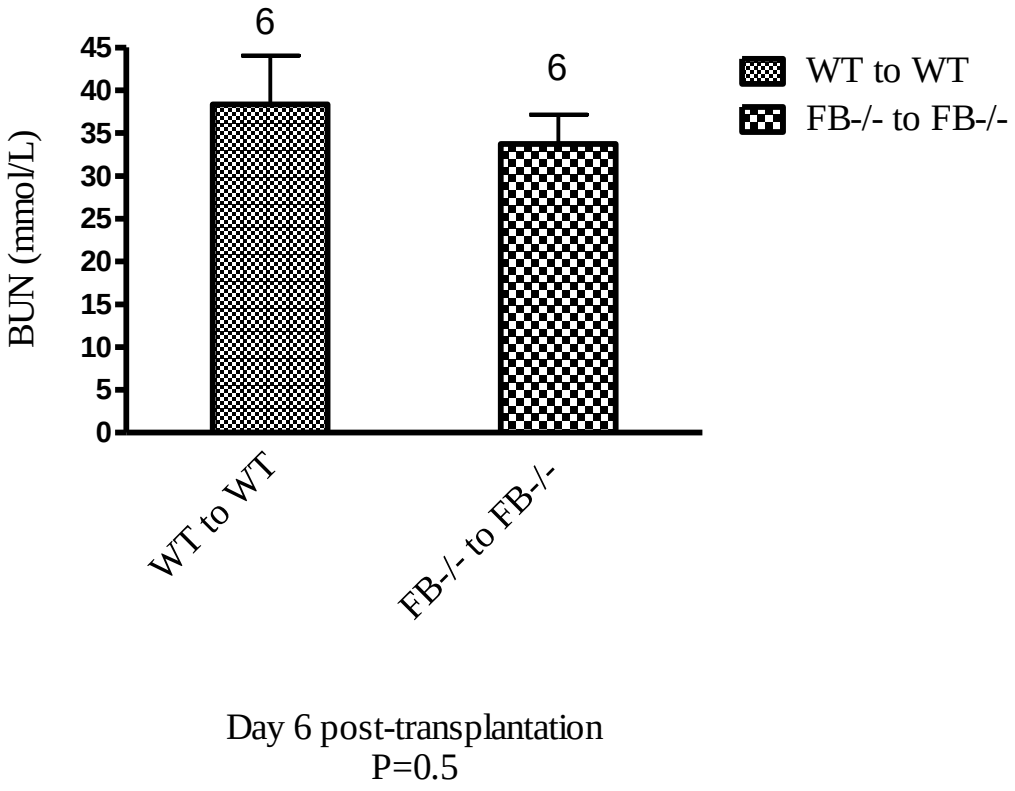
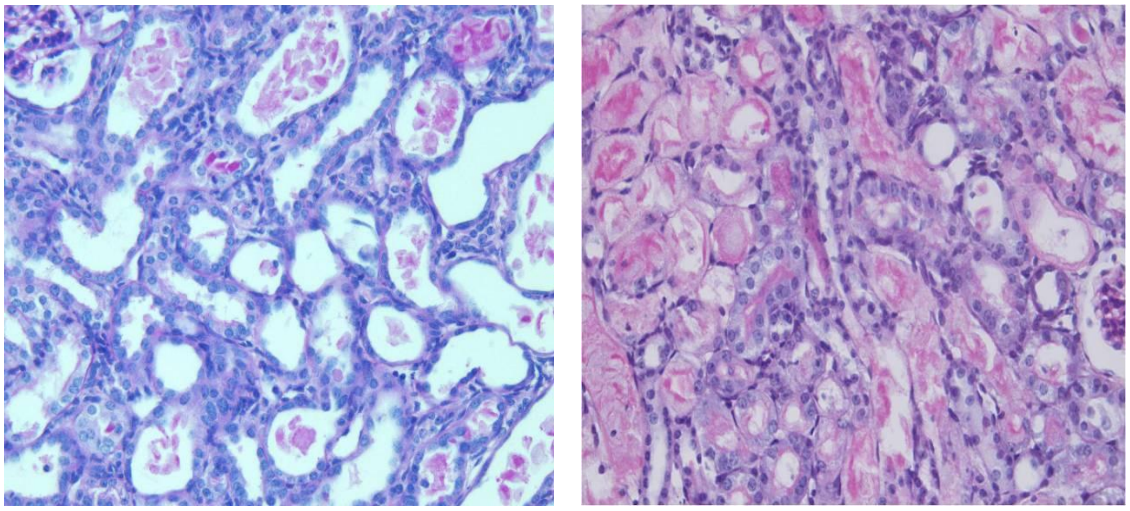


Figure 18. Influence of complement factor B on IRI in mouse kidney isografts. Comparison of renal function in WT to WT and FB-/- to FB-/- transplants 6 days after transplantation. Renal function was assessed by BUN measurement in the two transplant groups 6 days after the transplant procedure. The numbers above each bar represent the number of transplants in each group and the arrow bar indicates the standard deviation. Although the FB-/- group had slightly lower average BUN levels the difference between the groups was not statistically significant. P=0.5.

5.3.1 Kidney tissue damage in the absence of FB

To assess the level of kidney tissue injury following transplant IRI in WT and FB-/- groups, sections from the paraffin embedded kidney transplants were stained with PAS. Figure 19 shows the level of tissue injury in the 2 transplant groups.



WT to WT FB-/- to FB-/-

Figure 19. Kidney tubular necrosis in WT and FB-/- transplants. The above images show representative pictures from light microscopy of the transplanted kidney in WT to WT and FB-/- to FB-/- groups 6 days after transplantation. Both groups had significant levels of tubular damage, necrosis and cast formation (PAS staining, x25).

To assess complement deposition on the transplanted kidney tissue in this group, frozen sections from all kidney tissues as well as a non-transplanted FB-/- kidney as control were stained with C3d antibody as described above. A representative sample from WT and FB-/- transplant groups are shown in Figure 20.

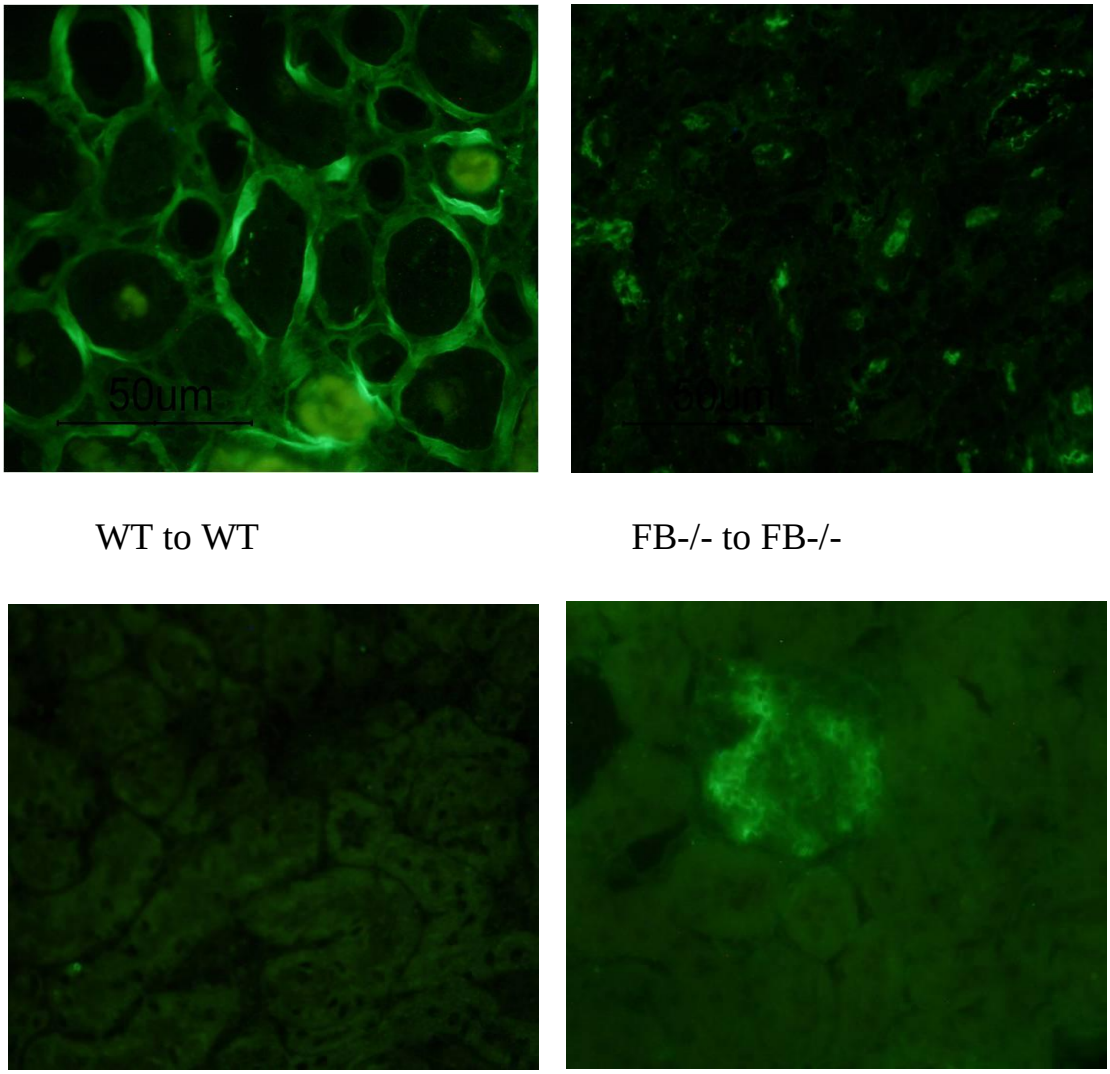


Figure 20. Immunohistochemical staining of the complement split product C3d in WT and FB-/- kidney tissues. The top panel shows the C3d deposition in the kidney of a WT mouse transplanted into a WT recipient (left) and a FB-/- mouse kidney transplanted into a FB-/- mouse (right). In the bottom panel on the left is the negative control staining (without primary Ab) of WT to WT transplant kidney and on the right is a section of a native uninjured FB-/- kidney stained for C3d. The transplant images are representative of 6 transplants performed in each group.

It was unexpected that despite almost similar levels of kidney injury noted by the BUN results and light microscopy of the kidney tissue from WT and FB-/- mice, C3d staining for these groups showed quite a different picture. As shown in Figure 20, the WT recipients had extensive C3d deposition in the tubular basement membrane whereas in the FB-/- transplant kidney (as well as the normal non-transplanted FB-/- kidney) there

was only some glomerular C3d deposition. This could be suggestive that the deposited C3d in the tubular basement membrane in the WT group is dependent on the alternative pathway activation. The absence of C3d staining in the kidney of FB-/- mice has previously been shown in native kidney IR model (Thurman et al., 2003); however in this study, the injury to the kidney was reduced in the FB-/- mice comparable with the reduced complement deposition. In my experiment, the fact that FB-/- mice had similar level of injury to the WT group can indicate that injury induced by complement in the transplant model, is perhaps not through the conventional route of MAC production. It can also be that factors inducing injury in the transplant model which do not exist in native IRI, such as cold and warm ischaemia, result in significant injury which reduces the effect of the alternative pathway activation. These results can strengthen the theory that factors of the LP of complement induce injury in the transplant model of IRI. Repeating the experiment with FB-/- mice with larger numbers is required to confirm the results.

5.4 Discussion

There are increased number of patients with end stage kidney disease who need a kidney transplant. However, this increase has not been met by the number of organs being donated, meaning that there is a widening shortfall between demand and supply. One of the most efficient ways to reduce this demand is to increase the survival of the available transplanted organs. It is well established that IRI is responsible for both acute kidney injury after transplantation as well as reducing long term graft survival. Activation of the complement pathway is known to play an important part in inducing this injury and not surprisingly a lot of interest has been shown in this subject in recent years, with the idea that modulating the responsible factor for complement activation in

this setting should reduce transplant organ damage and lead to longer graft survival. Protection of gene knock out animals deficient in various complement components as well as animals treated with complement inhibitors in various models of IRI have been very important in proving the role of complement cascade activation in IRI (Zhou et al., 2000, Thurman et al., 2003, Thurman et al., 2006, de Vries et al., 2003b, de Vries et al., 2003a). However, the initial triggering component for the pathway activation remains unclear.

The results of this study indicate that absence of MASP-2 enzyme which prevents LP activation significantly protects the organ from IRI. Deficiency of MASP-2 enzyme provides complete blockade of the LP in comparison to MBL deficiency, as ficolins are also able to activate MASP-2 (Matsushita et al., 2000, Matsushita et al., 2002). My results indicate that MASP-2 potentially by inhibition of the LP activation is critical in kidney IRI. The mechanism for induction of the LP activation could be the expression of damage associated molecules due to the IR stress during the renal transplant procedure. These putative DAMPs could then bind to the pattern recognition molecules such as MBL or ficolins, leading to activation of MASP-2 followed by the LP-induced cleavage of C3. The precise nature of these DAMPs and their presence at the hypoxia sensitive site remains to be identified.

That C4 deficiency did not show any protective effect in kidney IRI, while MBL/MASP-2 deficiency has been shown to be protective, raising the possibility that either the LP of complement can be activated independently of C4, perhaps via a different cleaving substrate, or it is possible that MBL/MASP-2 can induce tissue damage by a pathway that is independent of complement activation (Schwaeble et al., 2011). Indeed, in a recent study by van der Pol et al, MBL was shown to be able to induce renal tubular injury independent of complement activation in a rat model of

kidney IR (van der Pol et al., 2012). However, in this study systemic administration of monoclonal anti-C5 antibody or complement depletion with CVF were used and it is possible that the large size of these molecules prevented adequate penetration to the tubules, which is the site of complement activation that is mediated by IR insult (Park et al., 2001). Interestingly, Schwaeble et al have shown that the MASP-2 dependent C4-bypass activation of complement C3 is also dependent on blood clotting factor XI, since mice deficient of both C4 and factor XI lose the ability to activate C3 in a LP dependent fashion (Schwaeble et al., 2011). This suggests that elements of the coagulation cascade might be responsible for activation of complement.

Although in my experiment reduced C3d deposition in MASP-2 deficient animals post-transplantation indicates that the process of damage is complement dependent, there is still the possibility that MASP-2 is able to induce a complement-independent injury that is secondarily amplified by the deposition of complement on the injured tissue. In the latter case, MASP-2 should have an effect in the absence of C3, and double deficient animals (lacking MASP-2 and C3) should be further protected compared with C3 deficiency alone. If the effect of MASP-2 and C3 were not found to be additive, this would indicate an interdependent mechanism, in which MASP-2 led to the cleavage of C3 (bypassing C4). Experiment with C3-/-/MASP-2-/- animals would clarify this matter.

One of the limitations of this study is that MASP-2 was not identified within the kidney, despite its role in the generation of post-ischaemic damage of the renal tubules. Complement deposition on the renal tubules initiated by MASP-2 would require the presence of MASP-2 in association with MBL or ficolin at this locality. The absence of staining for MASP-2 in the post ischaemic tissue 6 days post transplantation would suggest the amount of MASP-2 was below the limit of detection at this time, which

could indicate a low threshold level of MASP-2 required for complement activation or is consistent with the short half-life for MASP-2 protein (26 minutes at 37 °C and pH 7.5) (Gal et al., 2005). It could also be that the most active period of injury is in the first 24-48h post-transplantation and the injury is established by day 6 when we have harvested the transplanted kidney. It would be indeed ideal to test the effect of transplantation by removing both native kidneys at the time of transplantation to reflect the real life situation of transplant dependent renal function from the start of transplantation. However, in our pilot studies the mice did not survive the injury if both kidneys were removed at the time of the transplant operation.

Inhibiting the LP prior to kidney transplantation could be an important therapeutic intervention for reducing IRI in this organ. Investigating the potential component that cleaves C3 independent of C4 would be very useful not only for helping us understand this bypass mechanism, but also due to the fact that it can open the way for possible therapeutic interventions.

6 Investigating the therapeutic benefit of Anti-MASP-2 antibody in kidney transplant IRI

Following the finding that absence of MASP-2 enzyme is protective in kidney transplant IRI; I investigated the therapeutic benefit of anti-MASP-2 use in this model. The idea was that inhibition of LP activation with an anti-MASP-2 antibody should theoretically reduce the IRI following kidney transplantation. If that would be the case, anti-MASP-2 antibody could be used to prevent kidney damage induced by transplantation.

6.1 Experimental design

Intraperitoneal (IP) administration of 0.6 mg/kg of the murine anti-MASP-2 antibody AbyD 04211 (provided by Omeros) has been shown to inhibit LP activation in the mouse serum within 6 hours of administration, with less than 10% of the activity recovering by 7 days (Schwaeble et al., 2011). Based on this data, 0.6 mg/kg of the monoclonal anti-MASP-2 antibody AbyD 04211 was injected intraperitoneally into 12 WT mice one day before they received a kidney transplant from a WT B6 donor. The same dose of the isotype control antibody was injected into a second group of 10 such mice one day before they received a WT B6 kidney transplant. The donor mice were not treated on the basis that MASP-2 enzyme is not produced in the kidney. As per the transplant protocol one native kidney was removed at the time of transplantation and the second native kidney was removed on day 5 post-transplantation. Kidney function was assessed by measuring the BUN in the serum from tail bleeding the recipient on day 6 (24 hours post second nephrectomy) and at day 7 (48 hours post second nephrectomy) using the serum obtained following cardiac puncture. The reason for checking the BUN at 48 hours after the second nephrectomy as well as at 24 hours was based on the maximal histological injury being described at 48 hours after the induction of IRI in native mouse kidney (Zhou et al., 2000). Three mice in the anti- MASP-2 treated group and 1 mouse in the isotype control treated group died between day 6 and day 7 and were excluded from the study. Both the anti-MASP-2 antibody and the isotype control were aliquoted after arrival from the company so that no added freezing and thawing was required. The experiment was carried out over a 6-week period with one to two transplants from each group (treatment and isotype control) performed on a single day.

6.1.1 Results

6.1.1.1 BUN measurement

As shown in Figure 21, no significant difference was observed between anti-MASP-2 and isotype control antibody treated group at day 6 (P= 0.63) or day 7 (P= 0.58). Although transplantations were done randomly and in each transplant day one anti-MASP-2 treated and one isotype control treated mouse was transplanted to reduce any potential bias related to the transplantation environment, there was significant variation in the range of BUN results between each group. The reason for this extreme variability and apparent lack of therapeutic effect was not clear. The absence of a therapeutic effect was thought to be possibly due to inadequate dosing or insufficient penetration of the antibody to the site of injury.

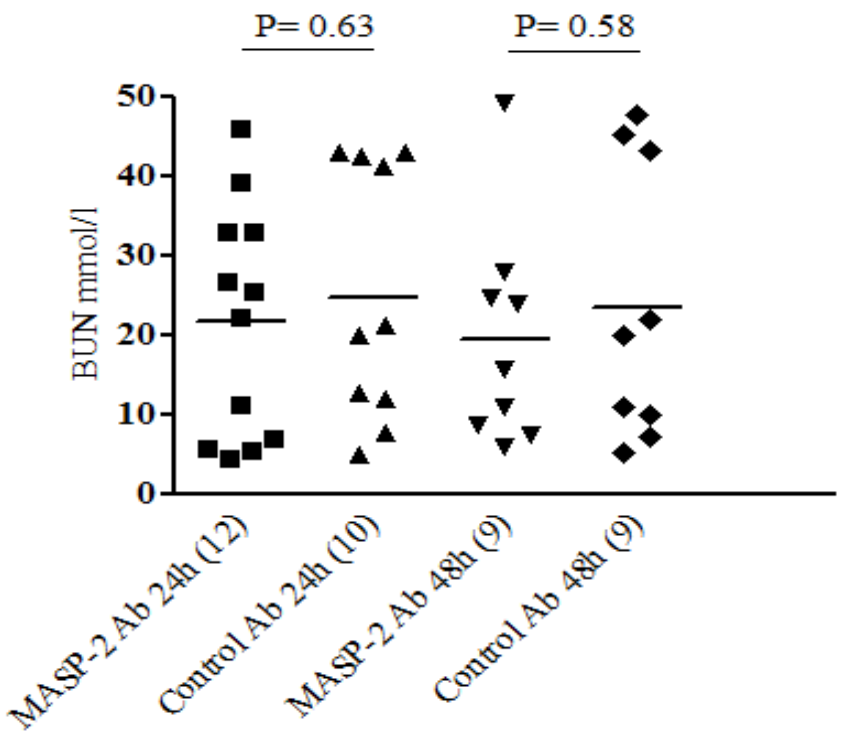
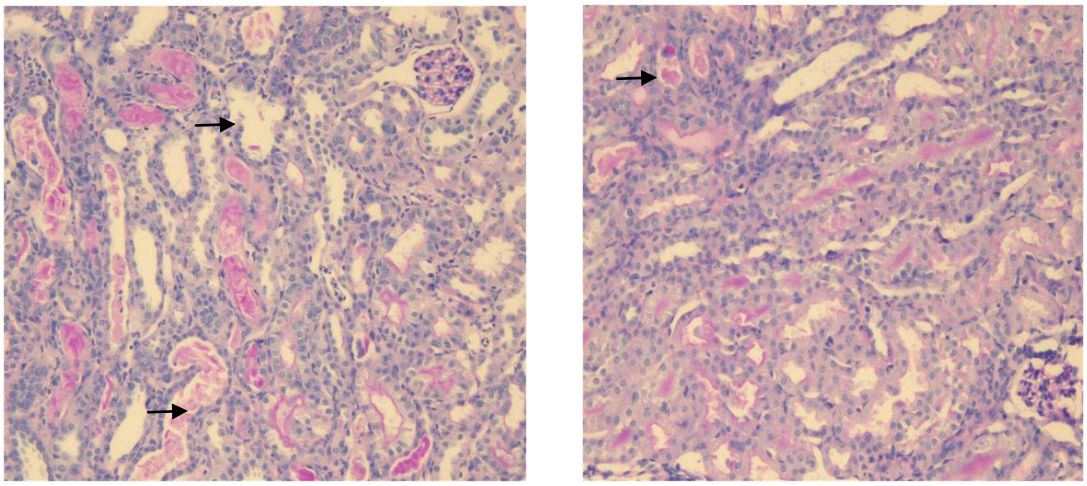


Figure 21. Treatment with anti-MASP-2 antibody does not protect from transplant IRI. Treatment with anti-MASP-2 or Control Ab pre-transplant and measurement of BUN at 24 and 48h post native nephrectomy (day 5) is shown. WT B6 transplant recipients were treated randomly with 0.6 mg/kg of anti-MASP-2 antibody or isotype Control Ab one day before kidney transplantation (IP injection). Transplantation was performed with 30 minutes of cold and 40 minutes of warm ischaemia time. One native kidney was removed at the time of transplantation and the second one was removed on day 5 post-transplantation. BUN levels were measured 24 hours and 48 hours after second native nephrectomy. Numbers in brackets indicate the number of transplants in each group. At 24 hours, the difference between the 2 groups had a P value of 0.63 and at 48 hours the P value was 0.58.

6.1.1.2 Histology

Histology sections of the paraffin embedded tissues from all the transplants in each group were prepared and stained with periodic Acid Schiff. Figure 22 shows a sample of kidney tissue from the anti-MASP-2 and isotype control treated antibody 48 hours after second nephrectomy (both samples are from mice with BUN of about 20 mmol/l).



Control Ab treated MASP-2 Ab treated

Figure 22. Histology of transplanted kidneys in recipients treated with anti-MASP-2 antibody and isotype control antibody. Light microscopy of kidney tissue from WT B6 mice treated with anti-MASP-2 antibody or isotype control antibody the day before transplantation. Both kidneys show evidence of tubular damage such as cast formation and necrosis (arrows). Magnification x25

Isograft group (number of transplants)	Mean severity score ± SD
Anti-MASP-2 Ab treated (9)	2.2 ± 0.78
Isotype control Ab treated (9)	3 ± 1.5

Table 8. Score of tubular injury in anti-MASP-2 and isotype control treated mice. Histology samples from anti-MASP-2 and isotype control treated mice, 48 hours after second native nephrectomy were reviewed and scored based on the tubular damage. The numbers in brackets show the number of transplants in each group. P=0.157

Similar to the kidney function data (measured by BUN levels) from the two treated groups, the histological data did not show a significant difference between the control treated mice and the anti-MASP-2 antibody treated recipients (Table 8). The histology samples correlated with the BUN results in both groups. Altogether, due to the wide range of BUNs in both groups, it was not possible to make a conclusion about the

results and repeat of the experiment was considered necessary to assess the effectiveness of the anti-MASP-2 treatment.

6.2 Discussion

Considering the significant protective effect that the absence of MASP-2 had on the kidney transplant IRI, the expectation was that the anti-MASP-2 antibody would provide some degree of protection from IRI in the therapeutic experiments.

There are several reasons that could explain why the anti-MASP-2 antibody treatment didn't show any protection in this experiment. One important factor is that the variation between the BUNs in each group has been high, leading to inconsistent injury in the kidneys which could be due to the surgical procedure or variation in induction of injury at different times through a lengthy experiment. To show a significant difference between 2 treatment groups with such high variation in injury, the sample size needs to be larger to detect a statistically significant difference. The other factor could be the dosing of the antibody which might not have been enough to inhibit the LP for the required period to prevent injury. Further experiments with dose titration needs to be performed to identify the optimal effective dose. The control antibody could have a therapeutic effect as well which could skew the result. In fact the Omeros Company later found that freeze and thawing the isotype control antibody, changes its characteristics in a way that it inhibits LP activation. Although all effort was made not to have the antibody go through freeze and thaw cycles, the main vial had to be thawed before being aliquoted and frozen before use. This could have changed the specificity of the control antibody and given it a protective effect (there was no change in specificity described with the anti-MASP-2 antibody following one freeze and thaw cycle). Presence of a sham operated group treated with a control such as normal saline would

have been appropriate in this experiment and would have clarified the true damage expected in the untreated mice.

Although application of anti-MASP-2 antibody in this experiment did not show a beneficial effect, protection of MASP-2-/- animals from IRI does show that LP via MASP-2 has a significant role in kidney transplant IRI. The protective effect of anti-MASP-2 treatment has been shown in other models of IR (Schwaeble et al., 2011). One other possible reason for the lack of protective effect in the transplant kidney model is the lack of adequate penetration of the antibody in the kidney tissue to prevent LP activation and induction of injury. Assessing the presence of antibody in the kidney at different time points following its administration can clarify its tissue penetration. Use of the recently developed small peptide which inhibits MASP-2 (Kocsis et al., 2010) would potentially overcome the tissue penetration barrier and could open up new therapeutic opportunities. Of course these are still animal models and would take some time to be translated to human experiments.

6.3 Conclusion

In conclusion, the results of this part of my study confirm the requirement for MASP-2 to produce full blown tubular injury following ischaemic insult of mouse donor kidney used for transplantation. The mechanism is independent of C4 but is associated with C3 deposition on the tubular surface. The precise trigger mechanisms leading to activation of MASP-2 and consequently to the cleavage of C3 and C5 will be of considerable interest to ongoing research, since it may identify new pathogenic targets on ischaemic tissue and reveal a non-conventional pathway of complement activation.

7 Effect of anaphylatoxins on cytokine production of monocytes and macrophages and their influence on adaptive immune response

7.1 Introduction

7.1.1 Rationale and Aim

The previous chapters of my thesis focused on the components that may activate the complement cascade following ischaemia-induced tissue stress, possibly through the exposure of damage associated molecular patterns on target epithelial or endothelial cells. I also focused on the possible effects of anaphylatoxin receptor C3a in mediating the subsequent inflammatory injury. With the second part of my thesis work, I aimed to assess in more detail, the cellular mechanisms that may be driven by this local complement activation which could ultimately contribute to IRI-mediated organ dysfunction in the context of graft rejection. I chose to assess the potential effect of the anaphylatoxins C3a and C5a on the production of pro-inflammatory cytokines by human monocytes and macrophages. This particular interest arose from recent publications suggesting an unexpected and important role for graft infiltrating IL-1β-producing monocytes in kidney transplant rejection (Girlanda et al., 2008, Zhang et al., 2005). Earlier studies had connected C3a and C5a and their desarginated forms (C3adesArg and C5adesArg) with the induction of IL-1β in human monocytes (Haeffner-Cavaillon et al., 1987, Okusawa et al., 1987). However, those studies raised controversies because at that time it was unclear if the preparation of the anaphylatoxins had been free of LPS - a strong inducer of IL-1β in monocytes (Arend et al., 1989). There were no subsequent studies on this subject and therefore the role of C3a and C5a in monocyte activation and their effector function, particularly in the setting of transplantation, was left largely unexplored.

In contrast to work with human monocytes, which can be easily purified in high numbers from freshly-drawn blood (de Almeida et al., 2000), isolating monocytes from non-manipulated (non-infected) mice is a time-consuming and expensive procedure ultimately leading to low numbers of cells for *ex vivo* experiments. I therefore first began addressing the role of C3a and C5a in monocyte activation by using blood-purified human monocytes (and macrophages) – with the future plan to confirm key findings in a mouse model of IRI/transplantation using pertinent complement component-deficient and/or monocyte-depleted animals.

Thus, I first aimed to investigate whether anaphylatoxins can induce or modulate the production of the key effector cytokine IL-1β by human monocytes and/or macrophages in a LPS-dependent or -independent fashion.

7.1.2 Monocytes

Monocytes are circulating blood leukocytes that are derived from bone marrow precursors and after circulating for an average time of 1-3 days, migrate into tissues and – depending on the activation stimulus – can develop into different subtypes (subpopulations) of macrophages with distinct functions (Locovei et al., 2006b). Monocytes also have the capacity to differentiate into dendritic cells (Pelegrin and Surprenant, 2006).

In humans, monocytes make up 10% of the blood leukocytes whereas in the mouse blood, they represent 4% of the leukocytes (Billaud et al., 2011). Monocytes have an important role in innate immune defense in response to invading microorganisms. They express a large variety and number of scavenger receptors that recognize pathogens and are able to produce inflammatory molecules such as complement factors, prostaglandins and reactive oxygen species as well as various inflammatory cytokines including IL-1β,

tumour necrosis factor (TNF), IL-6 and CXCL8 (chemokine ligand 8, known also as IL-8) (Madry et al., 2010). Monocytes are involved in key immunological processes including homeostasis and tissue repair (Kienitz et al., 2011, Chekeni et al., 2010) as well as modulation of T cell responses (MacVicar and Thompson, 2010, Mylvaganam et al., 2010). It is therefore not surprising that they also contribute to the pathogenesis of inflammatory conditions such as arthritis and atherosclerosis (Libby et al., 2008, D'Hondt et al., 2011). Monocytes were initially defined on the basis of their shape and later by their cytochemical characteristics (Woehrle et al., 2010). It was in the late 1980s that two subset of monocytes with distinctive phenotype and function were described based on their surface expression of CD14, a lipopolysaccharide co-receptor, and CD16 an Fc receptor. The majority of circulating monocytes were shown to express only CD14 but a small subgroup (of around 10%), expressed both CD14 and CD16 (Passlick et al., 1989). The number of CD16-positive monocytes has been shown to expand in several inflammatory conditions such as HIV disease, sepsis and rheumatoid arthritis as well as in haemodialysis patients with and without infection (Kawanaka et al., 2002, Fingerle et al., 1993, Thieblemont et al., 1995, Nockher and Scherberich, 1998); hence they were called pro-inflammatory monocytes. Further studies revealed that the CD16-positive monocyte consisted of two subpopulations that had different chemokine receptors: one with high and the other with low CD14 expression (Ancuta et al., 2003). In the following years different names were used to describe these various monocyte subpopulations until in 2010 a consensus nomenclature was proposed by a panel of monocyte biology experts who described monocytes in three distinct subtypes: 1) Classical monocytes which have high expression of CD14 but no CD16 expression ($CD14^{++}CD16^{-}$), 2) Intermediate monocytes which have high expression of both CD14 and CD16 ($CD14^{++}CD16^{+}$) and 3) Non-classical monocytes which have high levels of

CD16 and low levels of CD14 expression ($CD14^{+}CD16^{++}$). The latter 2 groups are sometimes referred to (in combination) as CD16-positive monocytes (Ziegler-Heitbrock et al., 2010). The different monocyte subpopulations differ in their expression of chemokine receptors, cytokine production and phagocytic activity and respond differently to infectious and inflammatory stimulations (Billaud et al., 2011). The classical monocytes have a strong phagocytic capability and produce antimicrobial proteins, whereas $CD14^{+}CD16^{+}$ monocytes have higher concentration of MHC class II and can act more effectively as antigen presenting cells (Passlick et al., 1989). $CD16^{+}$ monocytes also produce high amounts of inflammatory cytokines including TNF and IL-1β in response to stimuli such as bacterial lipopolysaccharides, viruses and immune complexes (Belge et al., 2002, Cros et al., 2010). While $CD14^{+}CD16^{+}$ cells express high levels of the chemokine receptors CCR5 and CX3CR1 (CX3C chemokine receptor 1), classical monocytes express chemokine receptor CCR2 and lower levels of CX3CR1 (Geissmann et al., 2003; Weber et al., 2000). CX3CR1 is a transmembrane G-protein coupled receptor that is expressed on a wide range of immune cells such as monocytes, natural killer cells, $CD4^{+}$ and $CD8^{+}$ T cells, mast cells and B cells and is involved in adhesion and migration of these cells (Corcione et al., 2009; Imai et al., 1997). CCR2 (chemokine receptor 2) and CCR5 (chemokine receptor 5) are chemokine receptors on leukocytes that facilitate migration and activation of the cells by which they are expressed on to their respective ligands at the site of infection/inflammation (Luster, 1998): monocyte chemo attractant protein-1 (MCP-1) for CCR2 and macrophage inflammatory protein 1α and 1β (MIP-1) and regulated on activation normal T cell expressed and secreted (RANTES) for CCR5. The variable expression of these receptors on different monocyte populations might contribute to their different migration pattern as well as their response to infectious stimuli (Mizuno et al., 2005).

It has been shown that monocytes are initially produced as the classical type in the bone marrow and after their release into the circulation they can differentiate to intermediate monocytes. Intermediate monocytes can then differentiate into non-classical monocytes. *In vitro* experiments have shown that different monocyte subtypes, when exposed to specific cytokines can transform into different subtypes of dendritic cells or macrophages (Sanchez-Torres et al., 2001, Rutherford et al., 1993). To investigate the role of monocytes in various *in vivo* models of disease, several studies have concentrated on defining the heterogeneous monocyte populations in animal models, mostly in mice (Strauss-Ayali et al., 2007). Initially surface markers such as CX3CR1, CCR2 and Gr1 (also known as Ly6C) were used to describe two main functional subsets of murine monocytes; 1- $CX3CR1^{(low)}CCR2^{+}Gr1^{+}$ and 2- $CX3CR1^{(High)}CCR2^{-}Gr1^{-}$ (Geissmann et al., 2003). Later, similar to human monocytes, a new nomenclature describing the mouse monocyte subpopulations was agreed, which divides them into three subsets based on their expression of Ly6C and CD43. Monocytes with high expression of Ly6C and low expression of CD43 ($Ly6C^{++}CD43^{+}$) represent the classical monocytes, high expression of both Ly6C and CD43 ($Ly6C^{++}CD43^{++}$) identifies the intermediate monocytes and low expression of Ly6C together with high expression of CD43 ($Ly6C^{+}CD43^{++}$) marks the non-classical monocytes (Ziegler-Heitbrock et al., 2010).

7.1.3 Macrophages

Macrophages are tissue-resident phagocytes derived from circulating monocytes and are present throughout the body (Iwamoto et al., 2010). They play an important role in maintaining tissue homeostasis by clearing apoptotic cells, producing growth factors and being involved in tissue remodeling and repair after injury (Eastgate et al., 1988,

Bryan et al., 2001). In addition to being a substantial source of inflammatory cytokines, macrophages have a broad range of pathogen recognition receptors (such as TLRs, complement receptor 3 (CR3) and mannose receptors) allowing them to effectively recognize pathogens and remove them through phagocytosis (Gordon, 2002). They also act as antigen presenting cells and influence the activation of specific B and T cells: After phagocytosis of pathogens and necrotic cells and the degradation of their protein constituents to peptides, macrophages are able to present them in conjunction with MHC II to T cells and initiate the adaptive immune response. T cell-derived IFN-γ further increases the expression of MHC II and co-stimulatory molecules on macrophages leading to their amplified ability for antigen presentation (Underhill et al., 1999, Barker et al., 2002). Macrophages present in the sub-capsular region of lymph nodes have been demonstrated to present viral particles as well as other antigens and immune complexes to B cells resulting in initiation of the humoral immune response (Junt et al., 2007, Carrasco and Batista, 2007, Phan et al., 2007).

Similar to monocytes, macrophages are a heterogeneous population and based on pathogen and cytokine exposure, they develop different functional abilities. Broadly, two functional groups named M1 and M2 are described: Exposure to microbial products such as LPS and a range of cytokines including IFN-γ, TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF) induces the M1 phenotype which is capable of producing inflammatory cytokines, reactive oxygen species and nitrogen metabolites as well as stimulating the Th1 response (Mantovani et al., 2009). The M2 phenotype refers to macrophages that are induced upon exposure to IL-4, IL-13, IL-10 and immune complexes. They have been shown to support Th2 response, have immunoregulatory functions and are involved in tissue repair and remodeling (Mantovani et al., 2004).

7.1.4 IL-1

Interleukin-1 (IL-1) is a pro-inflammatory cytokine with potent effects on many cell types alone or in combination with other cytokines and has a wide range of biological activities including regulation of the immune response and host defense (Church et al., 2008). Cloning of the IL-1 gene in 1984 (Auron et al., 1984) made a significant impact on the field of cytokine biology and stimulated many studies thereafter, investigating the role of this molecule in the pathogenesis of several inflammatory and auto-immune diseases such as rheumatoid arthritis (Eastgate et al., 1988), gout (Martinon et al., 2006), type 1 diabetes (Mandrup-Poulsen et al., 1986) and inflammatory bowel disease (McAlindon et al., 1998). Experiments to investigate the nature of IL-1 protein using macrophage cDNA library, led to the finding of 2 distinct proteins with IL-1 activity that were named IL-1α and IL-1β (March et al., 1985). Soon another protein was found as a member of IL-1 family named IL-1 receptor antagonist (IL-1Ra). IL-1Ra which was initially found in the supernatant of monocytes that were cultured on adherent immune complexes (Arend et al., 1985) and in the urine of febrile patients (Rosenstreich et al., 1988) is a naturally produced receptor antagonist that competitively binds to IL-1 receptor without initiating any intracellular response. In this way, IL-1Ra regulates the effect of pro-inflammatory components IL-1α and IL-1β (Arend et al., 1998) – an indication for the potency of IL-1 and the requirement of its tight control.

Both IL-1α and IL-1β are produced in response to stress or an inflammatory stimulus from cells of the innate immune system as precursor proteins known as pro-IL-1α and pro-IL-1β. IL-1α is active both as pro-IL-1α (Mosley et al., 1987) and following cleavage by calpain, which is a calcium dependent protease (Kavita and Mizel, 1995). Activation of IL-1β however requires the cleavage of the 31-33 kD precursor molecule by IL-1β converting enzyme, later named caspase-1, to the 17.5 kD mature form

(Thornberry et al., 1992). IL-1β is a multifunctional cytokine which either on its own or in combination with other cytokines can affect almost all cell types (Church et al., 2008) and influences a variety of physiological and pathological processes such as wound healing, mitogenic T cell stimulation, inflammation and sepsis (Schumann et al., 1998). IL-1β has also been shown to have a role in pathogenesis of cryopyrin-associated periodic syndrome consisting of auto-inflammatory disorders such as familial cold auto-inflammatory syndrome, neonatal onset multisystem inflammatory disease and Muckle–Wells syndrome in which a mutation in the NLRP3 inflammasome gene leads to overproduction of IL-1β (Church et al., 2006, Yu and Leslie, 2011). More recently, IL-1β has been shown to be important in modulating the adaptive immune response by inducing the development of pro-inflammatory Th17 cells from naive $CD4^+$ T cells (Acosta-Rodriguez et al., 2007, Wilson et al., 2007).

Monocytes and macrophages are the main sources of IL-β secretion. Various bacterial products such as LPS (by stimulating TLR4) and peptidoglycans as well as endogenous danger signals including extracellular ATP and uric acid have been shown to stimulate the production and release of IL-1β (Martinon et al., 2004, Mariathasan et al., 2006, Martinon et al., 2006).

7.1.5 Toll-Like Receptors

Toll like receptors (TLRs) comprise a family of pattern recognition proteins that are able to recognize a wide variety of pathogen-associated molecular patterns and damage-associated molecular patterns and play an important role in host defense against pathogenic infections as well as initiation of inflammation. They have structural homology to the Toll protein (hence being named after it) which was initially described

in Drosophila where they play a role in the immune response of the insect to fungal infections (Lemaitre et al., 1996, Hashimoto et al., 1988).

Extensive studies on TLR proteins have led to the identification of 11 TLR family members in humans and of 13 in the mouse (Robson, 2009). TLRs are expressed by immune cells such as dendritic cells, monocytes, neutrophils, T and B cells (Hornung et al., 2002, Zarember and Godowski, 2002) as well as tissue cells including epithelial cells in the renal tubules (Wolfs et al., 2002), intestine (Cario et al., 2000), the respiratory tract (Hertz et al., 2003) and endothelial cells in various organs (Zhang et al., 1999, Faure et al., 2000). The structure of TLRs consists of an extracellular domain that contains multiple leucine-rich repeats and a cytoplasmic domain which has strong homology with mammalian IL-1 receptor (Means et al., 2000). Later studies have identified that some of the TLRs including TLR3, TLR7, TLR8 and TLR9 are localized inside the cells in structures such as the endoplasmic reticulum and lysosomes and are able to detect foreign nucleic acid particles from viruses and bacteria and initiate an inflammatory response by inducing pro-inflammatory cytokine production (Blasius and Beutler, 2010). MyD88 (Myeloid differentiation primary response protein 88) is an important adaptor molecule which is involved in the signaling of all TLRs (except TLR3) (Medzhitov et al., 1998, Premkumar et al., 2010) as well as IL-1 and IL-18 (Adachi et al., 1998). Most TLRs mediate intracellular signaling events via the protein MyD88 to generate pro-inflammatory cytokines whereas MyD88-independent activation of TLRs predominantly controls the anti-viral interferon production (Kawai and Akira, 2006, Akira and Takeda, 2004). Stimulation of the TLRs by PAMPs for example derived from bacteria or viruses leads to activation of the NF-κB pathway which regulates the production of inflammatory cytokines such as IL-1, IL-6, IL-12,

TNF-α and induces the expression of co-stimulatory molecules including CD40 and CD86 thereby affecting both innate and adaptive immune response (Hou et al., 2008).

TLR4 is a member of the TLR family which responds to the LPS of gram-negative bacteria (Poltorak et al., 1998, Chow et al., 1999). TLR4 is expressed on monocytes and macrophages and its interaction with LPS on these cells has been the subject of extensive number of studies that aim at investigating how TLRs influence the innate and adaptive immune response in different inflammatory disease models as well as assessing therapeutic potentials by modulating its signaling (Hennessy et al., 2010). TLR activation on monocytes and macrophages is an important inducer of the inflammasome (explained in chapter 7.8) and of IL-1β production from these cells (Netea et al., 2008) and is discussed in more detail below.

7.1.6 Anaphylatoxins and TLRs

Complement and TLRs are key components of the innate immune system that in addition to providing the first line host defence, modulate the adaptive immune response. Considering their co-operative role in host defence and overlap in their stimulating factors, it is plausible that there is a crosstalk between these evolutionary old danger recognition systems. Several microbial and non-microbial factors have the ability to stimulate both of these systems of immunity. A good example is LPS, which, although well known for being a ligand for TLR4, is able to trigger complement activation (Bjornson and Bjornson, 1977, Morrison and Kline, 1977). A component of the yeast cell wall, zymosan, which activates both the classical and the alternative pathway of complement, has also been shown to stimulate TLR2 (Sato et al., 2003, Volman et al., 2005).

Using daf deficient (daf-/-) mice, Zhang and colleagues have demonstrated a functional interaction between complement and TLRs (Zhang et al., 2007). They noted that intraperitoneal injection of LPS in daf-/- mice resulted in significant elevation of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β in their plasma compared with the wild type control. In contrast, the level of IL-12 was higher in the wild type mice compared to the daf-/- mice. Further experiments using receptor antagonists and gene deficient mice showed that C5aR and to a lesser degree C3aR are responsible for the cytokine production of daf-/- mice which were treated with LPS (Zhang et al., 2007). The result of this experiment in combination with the findings (in the same paper) that WT mice treated with cobra venom factor (which activates and therefore depletes the serum from complement factors) or LPS alone did not have similar changes in the pro-inflammatory cytokine production, suggests that the effect on cytokines is the result of the synergistic effect of the anaphylatoxins in combination with TLR4. Interestingly, C5a had previously been shown to suppress TLR4 mediated production of IL-12 and IL-23 from inflammatory macrophages thereby reducing IL-12 dependent Th1 response both *in vitro* and *in vivo* (Hawlisch et al., 2005). Zhang and colleagues demonstrated that this effect is IL-10 dependent by showing increased IL-10 production in peritoneal macrophages exposed to both C5a and LPS (Zhang et al., 2007).

Taking into account that IL-6 has a role in Th17 cell polarization in the mouse, and the experiments by Zhang and colleagues which showed the synergistic effect of complement and TLR4 in the production of IL-6, Fang and colleagues looked into the effect of the mouse serum treated with LPS and C5a or cobra venom factor on cultured mouse T cells. They found that a higher percentage of these T cells develop into Th17 cells compared with T cells exposed to mouse serum activated with LPS, C5a or cobra venom factor alone (Fang et al., 2009). This effect was shown to be IL-6-dependent, as

neutralizing IL-6 in the T cell culture medium abrogated the differentiation of Th17 cells.

TLR activation has also been shown to have an effect on the production of certain complement components. Kaczorowski and colleagues have shown that stimulation of mouse macrophages with LPS result in increased factor B production (Kaczorowski et al., 2010).

Some pathogens have been shown to exploit the interaction of the TLRs and the complement system in a way that the immune response is either weakened or deviated in their favour (i.e. towards a more tolerogenic microenvironment). *Porphyromonas gingivalis*, a periodontal pathogen, has the ability to produce a protease which has C5 convertase activity and leads to production of C5a but not the membrane attack complex (Wingrove et al., 1992). Activation of TLR2 on macrophages by this pathogen in conjunction with ligation of the C5aR with C5a has been shown to result in increased cyclic AMP (cAMP) production which prevents the release of nitric oxide that is required for elimination of *P. gingivalis* leading to its survival (Wang et al., 2010).

The above investigations demonstrate the complex nature of crosstalk between the complement and TLR systems and their ability to modulate both the innate and adaptive immune response. They also reveal that the interaction between the two systems can sometimes be exploited by opportunistic pathogens to evade elimination by the immune response.

7.1.7 NOD-like receptors

The second vital group beside the TLRs in pathogen or danger recognition are the NOD-like receptors (NLRs) which as their name implies contain a nucleotide-binding oligomerization domain (NOD) (Inohara and Nunez, 2003). The NLRs are a group of

exclusively intracellular pattern recognition receptors that are able to sense danger signals such as PAMPs (for example, the RNA or DNA of cell-infecting viruses) and initiate an inflammatory response (Kanneganti et al., 2007). Since their recognition (Harton et al., 2002), 22 and 34 NLR proteins have been discovered in humans and mice, respectively (Jones et al., 2011). They are expressed in immune cells such as dendritic cells, monocytes, macrophages, T and B lymphocytes as well as in epithelial and endothelial cells (Kummer et al., 2007, Petterson et al., 2011). The structure of NLRs consists of three parts: 1) the effector N-terminal domain for protein-protein interactions which is either a caspase activation and recruitment domain (CARD), three baculovirus IAP (Inhibitor of Apoptosis Protein) repeats, or a pyrin domain (PYD) 2) a nucleotide-binding oligomerization domain (NOD) in the centre for nucleotide binding and self-oligomerization during activation and 3) the C-terminal part which consists of variable numbers of leucine-rich repeats (LRR) that are involved in sensing pathogens and modulate the NLR activity (Kanneganti, 2010). NLRs have been classified into three subgroups based on their N-terminal domains:

1. CARD-containing NLRs such as NOD1, NOD2 and the class II transactivator (CIITA). NOD1 and NOD2 are activated by bacterial peptidoglycans and are the inducers of mitogen-activated protein kinase (MAPK) and NF-κB signaling via activation of the serine-threonine kinase RICK (also known as RIP2) (Ogura et al., 2001, Kanneganti et al., 2007). Mutations in the NOD2 gene have been found in some patients with Crohn's disease (Hugot et al., 2001) and NOD1 polymorphism has been demonstrated to have a role in inflammatory conditions such as asthma (Hysi et al., 2005) and inflammatory bowel disease (McGovern et al., 2005) as well as host defense against *Helicobacter pylori* infection (Viala et al., 2004), suggesting that these molecules participate in mucosal immunity and homeostasis. CIITA is one of the main

regulatory factors for MHC II gene transcription, the absence of which leads to bare lymphocyte syndrome, a hereditary immunodeficiency condition (Reith and Mach, 2001).

2. IPAF (ICE (interleukin-1β -converting enzyme) protease-activating factor) and baculovirus inhibitor repeat (BIR)-containing NAIPs (neuronal apoptosis inhibitory protein) are a separate group of NLRs which are involved in apoptosis as well as inflammasome formation (Poyet et al., 2001, Liston et al., 1996).

3. The largest NLR subgroup is the NALPs (NACHT, LRR and PYD containing proteins) which has 14 genes recognized in humans (Tschopp et al., 2003). NALP1 and NALP3 (also known as cryopyrin), the most well studied molecules in this group, are known to use the ASC (apoptosis-associated speck-like protein containing a CARD) adaptor molecule to activate the inflammasome complex containing caspase-1, leading to production of the pro-inflammatory cytokines such as IL-1β and IL-18 (Martinon et al., 2001, Mariathasan, 2007, Mariathasan et al., 2006).

7.1.8 Inflammasome

Inflammasome is the term originally used to describe a high molecular weight intracellular complex that can activate inflammatory caspase enzymes and lead to production of pro-inflammatory cytokines such as IL-1β and IL-18 (Martinon et al., 2002). Similar to TLRs on the cell surface and within the endosomes, the inflammasomes have the ability to recognize PAMPs and DAMPs such as ATP, uric acid and DNA in the cell cytoplasm and initiate an inflammatory response (Figure 24) (Mariathasan et al., 2006, Martinon et al., 2006, Muruve et al., 2008).

Inflammasomes have been divided into four subtypes known as: NALP1, NALP3, IPAF and AIM2 inflammasome (Schroder and Tschopp, 2010) (Figure 23). NALP1 and

NALP3 inflammasomes consist of a sensor protein from the nucleotide binding and oligomerization domain NOD-like receptor (NLR) family, the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) and inflammatory proteases, caspase-1 and caspase-5 (Martinon et al., 2002). The CARD domain of the IPAF molecule directly interacts with the CARD domain of the procaspase-1 and can change it to the active form, caspase-1 in response to apoptotic and inflammatory stimulations such as endotoxic shock (Poyet et al., 2001, Li et al., 1995). AIM2 (absent in melanoma 2) is the most recent identified inflammasome that is activated by sensing double stranded DNA (dsDNA) from bacteria, viruses or self in the cytosole through its C terminal HIN-200 (hematopoietic interferon-inducible nuclear proteins with a 200-amino-acid repeat) domain (Fernandes-Alnemri et al., 2009, Hornung et al., 2009). AIM2 does not contain an NLR segment but has a pyrin domain which interacts with the pyrin domain of ASC molecule and is able to engage with procaspase-1 and similar to other inflammasome complexes, form the active caspase-1 and the pro-inflammatory cytokines IL-1β and IL-18 (Hornung et al., 2009, Burckstummer et al., 2009, Fernandes-Alnemri et al., 2009).

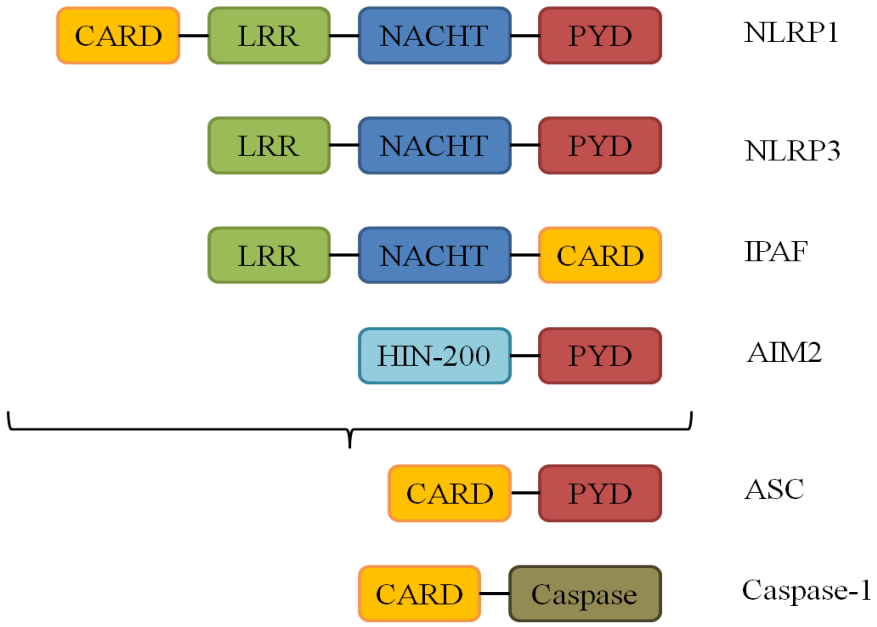


Figure 23. Infammasome subtypes. Schematic of the inflammasome subtypes NLRP1, NLRP3, IPAF, and AIM2 which in conjunction with ASC protein activate the caspase-1 complex. CARD: caspase recruitment domain; LRR: leucine-rich repeat; NACHT: nucleotide-binding and oligomerization domain; PYD: pyrin domain.

NALP3 is the most studied and best understood of the inflammasome complexes and has been shown to be involved in the pathophysiology of a variety of diseases. Viral RNAs and a wide range of bacterial products such as LPS, lipoteicoic acid and peptidoglycans when combined with ATP in millimolar concentrations are able to activate this inflammasome which in association with the ASC adaptor molecule leads to caspase-1 activation and production of the pro-inflammatory cytokines IL-1β and IL-18 (Kanneganti et al., 2006a, Kanneganti et al., 2006b, Sutterwala et al., 2006, Mariathasan et al., 2006). NALP3 is also responsible for sensing calcium pyrophosphate dehydrate and monosodium urate crystals in joints and inducing an inflammatory response by producing IL-1β and IL-18 (Martinon et al., 2006). As mentioned in the IL-1 section above (7.1.4), mutation in the NALP3 gene can cause a number of inherited auto-inflammatory disorders including Muckle-Wells Syndrome, chronic neurologic

cutaneous and articular syndrome and familial cold auto-inflammatory syndrome (Hoffman et al., 2001, Feldmann et al., 2002, Dode et al., 2002, Aganna et al., 2002) indicating the importance of this molecule in controlling inflammation.

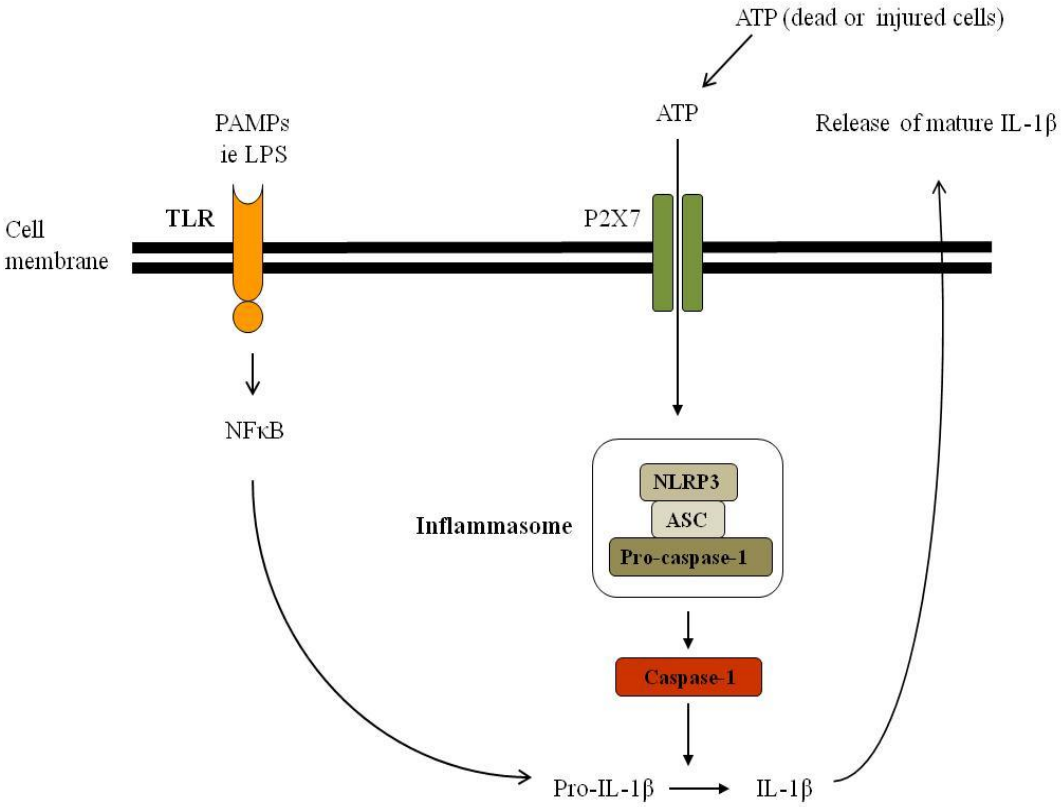


Figure 24. Activation of inflammasome leading to production of IL-1β. This diagram shows how stimulation of Toll Like Receptors in conjunction with another stimulus (ATP in this case) leads to activation of inflammasome and production of IL-1β in cells such as macrophages (in monocytes TLR stimulation alone is sufficient for IL-1β production (Netea et al., 2009)

7.1.9 Caspases

Caspases are a family of highly regulated proteolytic enzymes that are involved in a wide range of biological reactions such as apoptosis, inflammation, and cellular differentiation and remodeling (Crawford and Wells, 2011). They are produced as

inactive zymogens in the cell and undergo proteolytic processing (whereby a pro-segment which prevents the binding of the substrate to the enzyme is removed by proteolysis) or dimerization (where binding of the monomeric enzyme produces a dimeric structure that has more enzymatic activity) to become activated (Khan and James, 1998, Boatright et al., 2003, Donepudi et al., 2003). Caspases are classically also connected with programmed cell death in somatic cells (Kumar, 2007). Caspases involved in apoptosis are divided into 2 subgroups, the initiator (caspases-2, 8, 9 and 10) and the executioner (caspases-3, 6 and 7) caspases, with the initiator group sensing the death signal and activating the executioner group that cleave the required substrates leading to apoptosis (Boatright and Salvesen, 2003). Another group of caspases including caspase-1, 4, 5 and 14 in humans (1, 11 and 12 in the mouse) are known as inflammatory caspases because the substrates of the leading member of the group, caspase-1, are inflammatory cytokines, IL-1β and IL-18 (Nadiri et al., 2006). Caspase-5 is known to engage with the CARD domain of NALP-1, indicating that it might be involved in inflammasome activation through NALP-1.

Caspase-1 has been extensively studied in the past decade after its contribution to inflammasome activation was demonstrated (Martinon et al., 2002). The enzyme was originally discovered in experiments aimed at pinpointing the responsible enzyme that processes pro-IL-1β (Thornberry et al., 1992, Cerretti et al., 1992). Later, experiments using caspase-1-deficient mice confirmed the requirement of caspase-1 for IL-1β production, as these mice were unable to produce mature IL-1β and were resistant to endotoxic shock induced by injection of high dose LPS in the peritoneum (Li et al., 1995). Soon after, it was demonstrated that caspase-1 is required for activation of another pro-inflammatory cytokine, IL-18, which was known as IFN-γ-inducing factor (Gu et al., 1997) by showing that splenocytes from caspase-1-deficient mice have a

significantly reduced capability to produce IFN-γ after stimulation with LPS compared to the control group (Fantuzzi et al., 1998). As discussed in the inflammasome section (7.1.8), several inflammasomes activate caspase-1 with or without involvement of the ASC adaptor protein.

7.1.10 ATP and ion channels

As mentioned, TLR- and NLR-mediated assembly of the inflammasome and caspase-1 activation is one of the vital events required for IL-1β production in monocytes and macrophages. However, this alone is not sufficient in most cases to induce IL-1β and a second signal is required for successful cytokine production and that signal can be provided by ATP (adenosine-5'-triphosphate) and engagement of its receptor(s) (Figure 24).

ATP is the carrier of energy in all living organisms. It transfers the chemical energy released from nutrient metabolism to various cellular reactions that require energy. ATP was first discovered in 1929 by Karl Lohmann, a German chemist (Langen and Hucho, 2008). In the early 1940s, Fritz Lipmann (the 1953 Nobel laureate in Medicine) demonstrated that ATP is the common transporter of chemical energy and used the phrase ‘energy-rich phosphate bonds’ (Lipmann, 1941).

ATP is composed of an adenosine nucleoside which is bound to three phosphate molecules. Removal of the outermost phosphate molecule during hydrolysis, leads to formation of adenosine diphosphate (ADP) and release of energy which is used for cellular functions. The reverse of this reaction, binding of an inorganic phosphate to ADP with use of energy, forms ATP again.

With time, studies demonstrated that ATP is present in the extracellular space and plays an important role in various physiologic functions such as neurotransmission, cardiac

function, bone metabolism and muscle contraction (Holton, 1959, Forrester and Lind, 1969, Yu and Ferrier, 1993, Davies, 1963). Importantly, ATP has been recognized as DAMP and can cause inflammation with different mechanisms such as attracting neutrophils (Fredholm, 1997) or activating the inflammasome complex leading to production of inflammatory cytokines IL-1β and IL-18 (Cruz et al., 2007). More recently, ATP has been shown to have various immunomodulatory roles such as affecting T cell signaling (Schenk et al., 2008) and differentiation of Th17 cells (Atarashi et al., 2008).

Extracellular ATP exerts its effect by binding to two different types of receptors known as: 1) Ionotropic P2X receptors which are widely expressed in immune and non-immune cells of humans and have 7 subunits, P2X1 to P2X7 (Valera et al., 1994, Surprenant and North, 2009) and 2) G protein-coupled P2Y receptors (Webb et al., 1993).

The wide range of ATP function including its effect on inflammation and immune response has made P2X receptors interesting potential therapeutic targets in a variety of inflammatory, neurologic and cardiovascular disorders (Burnstock and Kennedy, 2011, Coddou et al., 2011). In both monocytes (Grahames et al., 1999) and macrophages (Mariathasan et al., 2006) activation of P2X7 receptor by ATP in conjunction with LPS has been shown to significantly enhance the production of IL-1β. In fact, in macrophages, second stimulation with ATP in addition to LPS has been suggested to be essential for production of IL-1β (Netea et al., 2009). Pannexin-1 hemi-channel which is expressed on most cells (Dubyak, 2009), is known to play a significant role in regulation of ATP efflux from various cell types (Ransford et al., 2009, Barbe et al., 2006), leading to activation of P2X7 receptor which as described above, contributes to IL-1β secretion.

As ATP engagement with its receptors has strong immunomodulatory functions, the extracellular availability of ATP needs to be regulated. This is achieved by the regulation of ATP cell efflux via specific channels, including the pannexin channels.

7.1.11 Pannexin Channels

Pannexins are a family of cell membrane channel-forming glycoproteins that were first described by Panchin and colleagues in the mammalian genome and the name pannexin (Panx) was suggested for these channels due to their wide distribution in animal species, where the Latin pan means all and nexus stands for connection (Panchin et al., 2000). Three pannexins have been identified: Panx1, Panx2 and Panx3. Panx1 is the most studied channel and its expression has been shown in a variety of human tissues such as the heart, skeletal muscle, ovary, testis, erythrocytes, small intestine, prostate, thymus, kidney, lung and brain (Baranova et al., 2004). Panx2 is expressed mostly in the brain and expression of Panx3 has been shown in chondrocytes, cochlea and epidermis (Baranova et al., 2004, Iwamoto et al., 2010, Wang et al., 2009, Celetti et al., 2010).

Molecules up to 1 KDa in size are able to cross the Panx1 channel (MacVicar and Thompson, 2010). One of the important functions of Panx1 which has been well studied is the control of ATP (507 D) release from cells (Locovei et al., 2006a) which acts as a paracrine signal to the surrounding cells. Release of ATP through Panx1 channel plays a role in a variety of cell functions such as initiation and propagation of calcium waves (Locovei et al., 2006b), regulation of vascular smooth muscle contraction (Billaud et al., 2011), sensation of taste through taste buds (Dando and Roper, 2009) and mucocilliary function of the airway epithelium (Ransford et al., 2009). Importantly, release of ATP from apoptotic cells mediated by Panx1 (Chekeni et al., 2010) acts as a ‘find me’ signal which attracts phagocytic cells including monocytes, macrophages and dendritic cells

which are responsible for removal of these dying cells (Elliott et al., 2009). As well as paracrine functions described above, Panx1 also influences the immune response; it has been shown that activation of caspase-1 of the inflammasome complex and release of the pro-inflammatory cytokine IL-1β induced by P2X7 receptor stimulation in macrophages, is mediated by Panx1 (Pelegrin and Surprenant, 2006).

The role of Panx2 has been less studied but so far it has been shown to be involved in neuronal differentiation (Iwamoto et al., 2010) and more recently absence of Panx1 and Panx2 (double knockout mice) has proven to lead to a smaller infarct size in a mouse model of ischaemic stroke, suggesting a combined role for these channels in inducing ischaemic brain damage (Bargiotas et al., 2011). The physiologic role of Panx3 has been the subject of several new studies. Panx3 promotes the differentiation of osteoblasts by acting as a Ca^{2+} channel in the endoplasmic reticulum as well as being able to form gap junctions and modulate the transmission of Ca^{2+} between cells (Ishikawa et al., 2011). Panx3 can also form a hemi-channel and release ATP into the extracellular compartment and modulate the intracellular cAMP levels which has been demonstrated to influence chondrocyte differentiation (Iwamoto et al., 2010).

7.1.12 Effector T cells

7.1.12.1 Th1/Th2 cells

The concept of $CD4^+$ T cells differentiating into distinct populations with exclusive effector capacities was first described in the 1980s by Mossman and colleagues, using mouse T cell clones (Mosmann et al., 1986). This group demonstrated two separate populations of mouse T helper cells, namely Th1 and Th2 based on their cytokine production profile. The presence of Th1/Th2 T-helper subtypes in humans was shown in the early 1990s. Using T cell clones from the peripheral blood of patients with allergies,

T cells showing Th1 and Th2 specificities were identified; the first was the main producer of IFN-γ in response to infectious stimuli and the second was shown to be allergen specific and mainly produced IL-4 (Wierenga et al., 1990, Parronchi et al., 1991). In addition to IFN-γ, Th1 cells also produce TNF and express the chemokine receptors CXCR3 and CCR5, whereas Th2 cells secrete IL-5 and IL-13 in addition to IL-4 and express surface markers CCR3 and CCR4 (Bonecchi et al., 1998, Sallusto et al., 1998, Loetscher et al., 1998, Sallusto et al., 1997). *In vitro* generation of Th1 and Th2 cells from naive precursor T cells was shown following stimulation with IL-12 and IL-4, respectively (Swain et al., 1990, Hsieh et al., 1993). In the following years, characteristics and functions of these two cell subtypes have been extensively studied and distinct roles have been identified for each group. Overall Th1 cells were found to initiate the response to intracellular viruses and bacteria and help cells of the innate immune system such as macrophages whereas Th2 cells were shown to be activated in response to extracellular stimuli such as helminthic infections and allergens and facilitate mast cell and basophil recruitment and activation (Abbas et al., 1996, Kawakami and Galli, 2002). The Th1/Th2 framework explained various disease processes in mice and in humans. Th1-related activity was demonstrated in autoimmune diseases including multiple sclerosis in humans (Selmaj et al., 1991) and its equivalent mouse model, experimental autoimmune encephalomyelitis (EAE)(Ando et al., 1989). Investigation of the animal model of autoimmune diabetes using non-obese diabetic (NOD) mouse indicated that the disease is mediated by Th1 cells (Campbell et al., 1991). In diseases such as asthma, however, Th2 responses were found to be predominant (Wills-Karp et al., 1998, Robinson et al., 1992). Further development in characterization of Th1 and Th2 was made possible with identification of the lineage-

specific transcription factors, t-box transcription factor T-bet (Szabo et al., 2000) and GATA3 (Zheng and Flavell, 1997), which respectively direct their differentiation.

For a long time the many aspects of immune function such as response to infections and allergic and autoimmune diseases was attributed to these two effector T cell subpopulations. However, some experimental findings particularly in autoimmune diseases such as immune-mediated arthritis and allergic asthma were not completely consistent with the Th1/Th2 paradigm and their occurrence have recently been attributed to a more recently defined T cell subtype known as Th17 cells (Steinman, 2007, Nakae et al., 2003, Hellings et al., 2003).

7.1.12.2 Th17 cells

Although for a long time Th1 and Th2 subtypes were thought to be the main components of the T-helper cell immune response, studies on autoimmune disease in mouse models including experimental auto immune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) led to the discovery of another T-helper linage later known as Th17 cells. The autoimmune process in these disease models was thought to be due to Th1 response and inhibition of IL-12 (which is required for Th1 activation) with antibodies or use of mice deficient in the p40 subunit of IL-12 attenuated the disease (Bright et al., 1998, Muller et al., 1998, Malfait et al., 1998, Gran et al., 2002). However, following the discovery of IL-23 cytokine and identifying that it contains the p40 subunit of the IL-12 molecule in addition to another subunit named p19 (instead of the p35 in IL-12) (Oppmann et al., 2000), further experiments using gene targeted mice were able to show that the development of both EAE and CIA were dependent on IL-23 rather than IL-12 (Cua et al., 2003). Aggarwal and colleagues then described that IL-23 induces the production of IL-17 from $CD4^+$ T cells (Aggarwal et al., 2003).

Even though IL-17 was known for over a decade to be produced by activated $CD4^+$ T cells, identification of Th17 cells as a distinct subset of T helper cells is a relatively recent finding (Harrington et al., 2005, Langrish et al., 2005, Park et al., 2005). Since their discovery, the influence of Th17 cells in adaptive immune response has been extensively studied and they are now acknowledged as key contributors to several inflammatory and autoimmune diseases (Kimura and Kishimoto, 2011). Investigation by Ivanov and colleagues identified the orphan nuclear receptor RORγt as the Th17 signature transcription factor required for differentiation of the Th17 cell linage (Ivanov et al., 2006). Following that, other groups identified IL-6 and TGF-β as the prominent cytokines necessary for differentiation of Th17 cells (Veldhoen et al., 2006, Bettelli et al., 2006); however, it was later demonstrated that these cytokines promote Th17 induction in the mouse system, but in humans, $CD4^+$ cells require IL-1β and IL-6 to develop and TGF-β has in fact an inhibitory effect on this process (Acosta-Rodriguez et al., 2007). IL-23 was initially thought to be required for Th17 differentiation, but it was later shown that despite having the ability to maintain the Th17 phenotype and IL-17 production, it does not affect the proliferation or survival of the Th17 cells (Stritesky et al., 2008).

Many of the effector functions of Th17 cells are mediated by their production of various cytokines and chemokines. The most studied cytokine produced by Th17 cells is IL-17A (also called IL-17). Detailed studies on IL-17 have identified five members in the family of this cytokine named IL-17A-F of which IL-17A and IL-17F have been shown to be produced by Th17 cells (Kawaguchi et al., 2004). Other important cytokines produced by Th17 cells include IL-22, IL-26 and IL-21 (Ouyang et al., 2008). Cytokines produced by Th17 cells have been shown to be important in host defense against extracellular pathogens, generating inflammation by recruiting cells of the

innate immune system particularly neutrophils and inducing autoimmune reactions (Miossec et al., 2009). More recently, Th17 cells and its cytokines have been demonstrated to have an immunoregulatory role in certain settings and provide protection from tissue destruction in autoimmune diseases such as inflammatory bowel disease and graft versus host disease (O'Connor et al., 2009, Yi et al., 2008, O'Connor et al., 2010).

It is important to note that despite the initial belief that $CD4^+$ T cells are the main producers of IL-17 (Fossiez et al., 1996), more recent studies have shown that other cells of the immune system such as macrophages, NK cells, γδ T cells and neutrophils as well as certain epithelial cells, are able to produce this cytokine (Cua and Tato, 2010). Unlike T cells, which require antigen specific signaling via the T cell receptor to produce IL-17, the other cells produce IL-17 independent of antigen stimulation, in response to cytokines such as IL-1β, IL-6, IL-23, TNF or DAMPs (Liu et al., 2004, Cua and Tato, 2010, Takahashi et al., 2008). Production of IL-17 by epithelial cells in the intestine has an important role in maintaining the integrity of the mucosal barrier (Kinugasa et al., 2000).

7.1.12.2.1 Anaphylatoxins and IL-17 production

Several recent studies have looked into the role of anaphylatoxins in the differentiation and regulation of Th17 cells in various animal disease models. In a model of experimental autoimmune encephalitis Liu and colleagues have shown that presence of both C3a and C5a is required for increased production of IL-17 which contributes to the pathogenesis of the disease (Liu et al., 2008). Stimulation of mouse peritoneal macrophages with C5a and LPS has been shown to induce IL-6 production which promotes differentiation of functional Th17 cells (from anti-CD3/CD28 stimulated T

cells) that are able to induce autoimmune arthritis and EAE in mice (Fang et al., 2009, Hashimoto et al., 2010). In contrast, in a mouse model of endotoxic shock Bosmann and colleagues have demonstrated that C5a has an anti-inflammatory effect and its addition to LPS-treated peritoneal macrophages leads to a reduction in IL-17 production in a dose dependent manner (Bosmann et al., 2012). They showed that this effect is due to induction of IL-10 which strongly inhibits IL-17 production.

The differential effect of anaphylatoxins on Th17 cell differentiation has been described in a mouse airway hyper responsiveness (AHR) model where deficiency of C5aR resulted in increased production of IL-17 and development of severe asthma whereas C3aR deficiency was associated with reduced number of IL-17 producing Th17 cells and presentation of less severe form of asthma (Lajoie et al., 2010). C3a-dependent induction of Th17 cells leading to increased production of IL-17 has been described in other studies using the AHR mice model (Bera et al., 2011, Mizutani et al., 2012). Interestingly, in a mouse model of sepsis induced by cecal ligation and puncture, C5a was shown to induce IL-17 production from γδ T cells (Xu et al., 2010). This effect was dependent on the presence of DCs and production of IL-6 and TGF-β from these cells.

Studies investigating the role of anaphylatoxins on Th17 development in humans are limited. High levels of serum C5a has been shown in patients with age related macular degeneration (AMD) (Reynolds et al., 2009), leading to increased production of IL-6, and IL-1β from monocytes which in turn induces the production of IL-17 and IL-22 from $CD4^+$ T cells, a process that could be related to the pathogenesis of the disease (Liu et al., 2011).

These studies demonstrate that anaphylatoxins do influence the differentiation of Th17 cells and production of its main cytokine, IL-17. However, their differential effect in different disease models make a universal approach to their modulation complicated. As

discussed above, other immune cells have the ability to produce IL-17, a fact that should be taken into consideration when inhibition of its effect is being considered.

7.1.12.3 Regulatory T cells

Regulatory T cells (Tregs) are a population of T cells that are known to suppress and regulate the activity of the immune response. They are broadly divided into two groups: 1) natural Tregs (nTregs) which originate in the thymus and 2) inducible Tregs (iTregs) which acquire regulatory function in the periphery (Adalid-Peralta et al., 2011). nTregs are derived from a distinct population of naive $CD4^+$ T cell precursor with a different T cell receptor repertoire than the conventional naive $CD4^+$ T cells (Romagnoli et al., 2002) and posses suppressive capacity following their production in the thymus. They express the key transcription factor forkhead box P3 (FoxP3) as well as CD25 which is the IL-2 receptor-α chain and the surface marker cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Fontenot et al., 2003, Sojka et al., 2009, Hori et al., 2003). The iTregs are, however, generated in the periphery from naive $CD4^+$ cells under certain environmental conditions where there is T cell receptor stimulation as well as presence of IL-2 and TGF-β cytokines. Presence of these factors induces the expression of FoxP3 and CD25 on the T cells and gives them suppressive capacity. Induction of iTregs *in vitro* has been demonstrated when naive $CD4^+$ T cells were stimulated with antigen in the presence of TGF-β (Chen et al., 2003, Fu et al., 2004).

7.1.12.4 Other T helper cells

Other than the 4 distinguished T helper cell lineages Th1, Th2, Th17 and iTregs which develop from naive $CD4^+$ T cells, a number of T cell subsets have been identified in the past decade which do not belong to either of these categories. Follicular helper T cells

are one of these T cell subsets which are known to reside in the germinal centre of the peripheral lymph nodes, produce IL-21 and influence B cell function (Nurieva et al., 2008). They express the chemokine receptor, CXCR5, and have a key role in differentiation of B cells and their antibody production (Kim et al., 2001, Breitfeld et al., 2000). Differentiation of follicular helper T cells is regulated by the transcription factor Bcl6 (B cell lymphoma 6) the expression of which is mediated by IL-6 and IL-21 (Johnston et al., 2009, Nurieva et al., 2009).

Production of IL-9 from naive $CD4^+$ T cells following stimulation with TGF-β and IL-4 was initially described by Schmitt and colleagues (Schmitt et al., 1994). It was later suggested that these IL-9 producing cells belong to a distinctive T cell linage named Th9 cells (Dardalhon et al., 2008, Veldhoen et al., 2008). Interferon-regulatory factor 4 (IRF4) and transcription factor PU.1 have been shown to be essential for the development of Th9 cells (Staudt et al., 2010, Chang et al., 2010). IL-9 has a role in host defense against helminthic infections and mediates an inflammatory response in allergic asthma (Cheng et al., 2002, Nicolaides et al., 1997, Tan and Gery, 2012).

Another subset of T helper cells named Th22 which secret IL-22 and TNF-α has been identified in the skin infiltrates of patients with inflammatory skin diseases such as psoriasis (Eyerich et al., 2009). They have an epidermal homing characteristic and are involved in epidermal inflammation and wound healing (Duhen et al., 2009, Boniface et al., 2005). More recently they have been shown to have an important role in host defense against enteropathogenic bacteria (Basu et al., 2012).

T cell differentiation to various subtypes continues to be the subject of many investigations and there is the potential for discovery of further subsets.

7.1.13 Kidney transplant rejection

Since the early 1990s the histopathological appearance of kidney transplant tissue following rejection has been classified under Banff criteria. Banff is the international standardized criteria for histological diagnosis and classification of kidney transplant pathologies set by a group of renal histopathologists, nephrologists and transplant surgeons who initially met in Banff, Canada, in 1991 with the purpose of facilitating international comparison of data on kidney transplant rejection and to ultimately improve patient care (Solez et al., 1993). Since then the criteria (summarized in table 9) is revised every few years and the latest was in 2011 (Mengel et al., 2012).

1. Normal
2. Antibody-mediated rejection. Histological grade: I. ATN-like, C4d +, minimal inflammation II. Capillary- margination and/or thromboses, C4d + III. Arterial, C4d +
3. Borderline changes ('Suspicious' for acute cellular rejection): No intimal arteritis is seen but there are foci of mild tubulitis (1–4 MN cells/tubular cross-section)
4. Acute/active cellular rejection (T cell-mediated). Histological grade: - 1A: Cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of moderate tubulitis (>4 MN cells/tubular cross section or group of 10 tubular cells) - 1B: Cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of severe tubulitis (>10 MN cells/tubular cross-section or group of 10 tubular cells) - 2A: Cases with mild to moderate intimal arteritis - 2B: Cases with severe intimal arteritis comprising >25% of the luminal area - 3: Cases with transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation
5. Chronic/sclerosing allograft nephropathy: Fibrosing changes in the allograft, with or without features of true alloimmune injury to the graft. Histological grade: I. Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific changes suggesting chronic rejection (mild) II. Moderate interstitial fibrosis and tubular atrophy (a) or (b) (moderate) III. Severe interstitial fibrosis and tubular atrophy and tubular loss (a) or (b) (severe)
6. Other: changes not considered to be due to rejection.

Table 9. Banff diagnostic categories for kidney transplant rejection. There might be an overlap between different categories on histology samples (Racusen et al., 2003). MN: mononuclear cells; ATN: Acute Tubular Necrosis.

In broad terms, kidney transplant rejection is categorized into antibody-mediated rejection (AMR) or T cell-mediated rejection, each of which can present in acute or chronic form and have different degrees of severity (mild, moderate or severe rejection). AMR (also known as humoral rejection) occurs due to the presence of anti-donor antibodies leading to damage of endothelial cells in microcirculation potentially through complement activation and infiltration of leukocytes (Sis et al., 2009). Presence of C4d deposition in peritubular capillaries of the kidney tissue in the biopsy samples has been one of the hallmarks of AMR (Colvin, 2007). However, more recently it has been recognized that in some cases (particularly when chronic), AMR can occur with negative C4d staining (Sis et al., 2007, Miura, 2007) perhaps due to the presence of non-complement fixing antibodies or fading of deposited C4d. This issue has been raised in the latest Banff meeting in 2011 suggesting a need for a new diagnostic criteria for AMR independent of C4d which is expected to be developed for the next meeting in 2013 (Mengel et al., 2012). In addition to positive C4d staining, infiltration of neutrophils and/or mononuclear cells in the peritubular capillaries, features of acute tubular injury and presence of thrombi and fibrinoid necrosis are elements suggestive of AMR (Sis et al., 2010).

In T cell-mediated rejection, there is interstitial infiltration of T cells associated with areas of tubular inflammation (tubulitis) the intensity of which defines the grade of rejection. Inflammation of the vessel wall is another feature of T cell-mediated rejection which can present as mild to moderate inflammation in the intimal layer known as intimal arteritis to involvement of the whole vessel wall (transmural arteritis) where inflammation extends to the medial smooth muscle cells and can lead to necrosis and infiltration of lymphocytes (Sis et al., 2010). AMR and T-cell mediated rejection can sometimes coincide.

Several studies have looked into identification of the types of cellular infiltrate in kidney transplant rejection with the aim of predicting prognosis and tailoring treatment. Presence of $CD4^+$ and $CD8^+$ T cells in kidney allograft rejection was originally defined by Bolton et al (Bolton et al., 1989) and is a well established phenomenon in T cell-mediated rejection. In addition to T cells, monocyte and macrophages are known to have a significant role is acute and chronic allograft rejection by stimulating T cells (as antigen presenting cells) as well as producing pro-inflammatory cytokines such as IL-1, IL-12, IL-18, TNF-α and IFN-γ (Wyburn et al., 2005). Eosinophils and B cells have also been demonstrated in some cases of graft rejection and are associated with steroid resistance and worse prognosis (Sarwal et al., 2003, Nankivell and Alexander, 2010). Presence of NK cells has been more recently described in AMR with suggestions that they can be used as a diagnostic tool for rejection episodes (Hidalgo et al., 2010). I have discussed the role of various T cells and monocyte/macrophages in kidney allograft rejection in more detail in sections below.

7.1.13.1 Monocytes and macrophages in transplanted kidney

Presence of monocytes in transplanted kidneys has been described to be associated with poor transplant outcome since the early 1980s (Harry et al., 1984). At the time, non-specific esterase reaction was used to identify monocytes. CD14 as well as CD68 which were later identified as markers for monocytes and macrophages are now used to assess the infiltration of these cells in kidney tissue biopsies and the cells have been referred to as monocytes or tissue macrophages in different studies. In a study of 154 renal allograft biopsies, Dooper and colleagues demonstrated that staining for peritubular CD14 offers a valuable marker for diagnosis of acute rejection with positive predictive value of 95% and a negative predictive value of 47%, when compared with the original

diagnosis made on the samples using the Banff criteria (Dooper et al., 1994). Using CD68 as a marker of monocytes/macrophages, it has been shown that presence of these cells in the transplant kidney biopsy is not only a marker of rejection but also predictor of poor long term graft outcome in studies performed up to 4 years after transplantation (Ozdemir et al., 2002, Tinckam et al., 2005). Macrophages were also suggested to play a key role in acute vascular rejection in kidney transplants, being identified as the predominant cell types in intimal arteritis which is diagnostic of this condition (Matheson et al., 2005). However, this view was later challenged by Kozakowski and colleagues, who described that although monocytes are present in intimal arteritis, their presence is not associated with a humoral response and their infiltration of the intima did not predict a worse outcome when compared to levels of T cell infiltration observed (Kozakowski et al., 2009).

7.1.13.2 T cells in kidney transplant rejection

T cells are the key elements in the process of kidney transplant rejection (Bolton et al., 1989). Presentation of donor alloantigens by either the donor (direct pathway) or the recipient (indirect pathway) APCs to recipient T cells in the draining lymph nodes and the spleen results in clonal expansion of alloreactive T cells which then differentiate to $CD4^+$ or $CD8^+$ T cells and initiate an effector immune response (Jiang et al., 2004). $CD4^+$ T cells are not only important in activation of B cells that produce alloantibodies, but also assist the induction of delayed type hypersensitivity responses by macrophages whereas $CD8^+$ T cells induce apoptosis and cell death in the donor organ (Sayegh and Turka, 1998). These reactions collectively lead to graft rejection and damage of the transplanted organ.

Within the subset of $CD4^+$ T cells, Th1 population has been shown to have a more prominent role in kidney transplant rejection. Discovery of other subpopulation of T cells, in particular Th17 cells, has led to the investigation of their role in various immunological reactions including transplant rejection. As the contribution of Th17 cells to transplant rejection is relevant to my work, I have explained it in more detail below.

A few recent experimental studies have investigated the role of Th9, Th22 and follicular T cells in transplant rejection (Liu et al., 2013) which is the beginning of a potentially interesting field.

7.1.13.3 Th-17 cells in kidney transplantation

Production of IL-17 was shown to contribute to transplant rejection before Th17 cells were described, when Vankooten and colleagues demonstrated that kidney transplant biopsies from patients with graft rejection had positive immunofluorescent staining for IL-17 compared to patients with no rejection who had negative staining for IL-17 (Van Kooten et al., 1998, Strehlau et al., 1997). Later, presence of IL-17 was reported in the transplanted kidney tissue in a rat model with acute borderline transplant rejection as well as in human kidneys with subclinical kidney rejection (Hsieh et al., 2001). Hsieh and colleagues also demonstrated the existence of IL-17 mRNA in the mononuclear cells obtained from the urinary sediment of patients with borderline subclinical kidney transplant rejection which was not present in the control group indicating that IL-17 measurement in the urine could have a predictive value for kidney transplant rejection (Hsieh et al., 2001).

In addition to a higher number of neutrophils and lymphocytes, increased levels of IL-17 mRNA and protein have been demonstrated in the bronchioalveolar lavage of lung

transplant patients with acute rejection suggesting its contribution to the pathogenesis of rejection (Vanaudenaerde et al., 2006). Increased levels of IL-17 as well as IL-23 have also been shown in liver transplant patients with acute rejection (Fabrega et al., 2009).

Although it was initially presumed that $CD4^+$ T cells are the source of IL-17 in the rejecting kidney transplant tissue, more recent studies indicate that despite presence of high levels of IL-17 in rejecting transplant tissue compared to non-rejecting samples, the source of this IL-17 is not necessarily always the infiltrating $CD4^+$ T cells. In one study, assessment of 49 human kidney transplant biopsy samples with acute rejection found high number of IL-17^+ cells which were mostly neutrophils and mast cells (Yapici et al., 2011). Loverre and colleagues have demonstrated that renal tubular epithelial cells produce IL-17 in acute antibody mediated rejection which interestingly co-localizes with C4d deposition in peritubular capillaries (Loverre et al., 2011). They have also shown that stimulation of proximal tubular epithelial cells with C3a *in vitro*, results in expression of IL-17 in these cells which could be the mechanism by which they produce IL-17 in AMR.

This potentially important role of IL-17, produced from cells other than classic $CD4^+$ Th17 T cells has also been investigated in other animal models of transplantation. In a mouse heart transplant model, production of IL-17 from γδ T cells rather than $CD4^+$ T cells was shown to have a key role in acute rejection and absence of IL-17 (using IL-17-deficient mice) resulted in expansion of regulatory T cells, suggesting an inhibitory role of IL-17 in the development of these latter cells (Itoh et al., 2011).

The above studies suggest strongly that IL-17 does have a role in the pathogenesis of transplant rejection; however, the source of IL-17 in this setting as well as the target cells may vary and warrants further exploration.

Based on review of this literature, I proposed that IL-β produced by monocytes and macrophages in response to anaphylatoxins with or without TLR signaling, is a potent effector mechanism that could in part explain the complement-mediated inflammatory reaction following metabolic stress. I also hypothesized that if ATP as an additional signal is required for monocyte and macrophage activation, Panx1 channel has a role in its release into the extracellular space. For technical reasons explained earlier in this Chapter, I used human rather than mouse monocytes as the preferred cell type, and I used LPS for TLR4 stimulation as an established model for TLR-mediated signaling.

Also, taking into account the combined role for anaphylatoxins, monocytes and IL-17 producing cells in kidney transplant rejection, my plan was to investigate if the effect of anaphylatoxins on monocytes/macrophages (with or without TLR activation) would result in production of inflammatory cytokines which could affect the T cell response in kidney transplant rejection.

8 Materials and Methods

8.1 Materials

8.1.1 Plastic ware

24-well and 12-well cell culture plates were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany).

8.1.2 Chemicals and reagents

EDTA to detach cells for co-culture was from Fisher Scientific and Tryptan Blue to assess cell viability (under microscope) was obtained from Sigma Aldrich (Saint Louis, MO, USA). Tween 20 used in wash buffers was from Calbiochem (Darmstadt,

Germany). Ficol for separation of peripheral blood mononuclear cells was purchased from Amersham (New Jersy, USA). Propidium Iodide to detect cell viability (with flow cytometry) was purchased from BD Biosciences. For intracellular staining phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma and monensin (420701) from Biolegend.

8.1.3 Media and buffers

RPMI GlutaMAX 1640 media, Sodium Pyruvate, non-essential amino acids (NEAA) and Penicillin-Streptomycin were all purchased from Gibco, Invitrogen, PBS (phosphate buffered saline) and FCS (fetal calf serum) used in the cell culture experiments were from Hyclone (Utah, USA).

Monocyte and T cell culture media:

RPMI GlutaMAX media, 10% FCS, 1% penicillin-Streptomycin (100 units/ml penicillin and 100 µg/ml streptomycin) – the same media was used for both monocytes and T cells.

Macrophage culture media:

RPMI GlutaMAX, 10% FCS, 1% penicillin-Streptomycin (100 units/ml penicillin and 100 µg/ml streptomycin), 1% NEAA (non-essential amino acids), 1% sodium pyruvate and 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF).

Bead separation buffer:

PBS + 0.5% FCS + 1.5 mM EDTA

ELISA wash buffer:

PBS + 0.05% Tween

FACS (fluorescence-activated cell sorting) buffer:

PBS + 5% FCS

8.1.4 Antibodies and cytokines

Anti-CD3 (OKT-3) was prepared in-house from a specific hybridoma line; anti-CD28 was from BD Biosciences, recombinant IL-6, IL-1β and IL-23 were purchased from R&D systems. Recombinant human IL-2 was a gift from Dr. Christine Pham (Washington University in Saint Louis, MO, USA). Anti-IL-4 and anti-INF-γ were from eBioscience (Hatfield, UK).

The following mouse monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used for flow cytometry in this study: FITC-labeled anti-C5aR Ab (CD88 S5/1, sc-53795) and FITC-IgG Ab as isotype control (sc-2856), both from Santa Cruz biotechnology. PE-conjugated human anti-C3aR Ab (Biolegend, 345804) and PE-conjugated mouse anti-human-IgG as isotype control (BD Biosciences, 400314).

For pannexin-1 studies, polyclonal rabbit anti-pannexin antibody from Abcam Plc (Cambridge, UK) (ab60098) was used as well as polyclonal rabbit IgG as isotype control. Both antibodies were PE-conjugated using Zenon PE rabbit conjugating kit from Invitrogen (Z-25355) according to the manufacturer's instructions.

For immunohistochemistry, anti-CD4 (clone BC/1F6), anti-IL-17 (polyclonal), and anti-C3a (clone 4H3), all purchased from Abcam Plc, were used. Anti CD68 antibody was also from abcam.

8.1.5 Recombinant proteins, protein inhibitors and receptor agonists and antagonists

Recombinant human C5a (234397) was purchased from Calbiochem (San Diego, USA) and serum-purified human C3a (A118) was obtained from Comptech (Texas, USA), C3a receptor agonist (C4494) and LPS (lipopolysaccharide) were from Sigma Aldrich.

Caspase-1 inhibitor (400015) was from Calbiochem, ATP (A1852), oxidized ATP (oxATP, A6779) and Carbenoxolone (C4790) were from Sigma Aldrich. Recombinant Human IL-1 receptor antagonist (C-61122) was from Promokine (Heidelberg, Germany). M-CSF was purchased from Gibco, Invitrogen.

8.2 Methods

8.2.1 Purification of T cells and monocytes by magnetic bead separation

Fresh blood was obtained from healthy volunteers with the approval and in accordance of the King's College Ethics Committee guidelines (Reference No: 06/Q0705/20) and collected in sodium heparin containing tubes (BD Vacutainer, BD Biosciences). The blood was diluted in 50 ml Falcon tubes 1:1 with 1 x PBS. 30 mls of the diluted blood was then layered using a pipette over 15 mls of ficol and then centrifuged at 1600 rpm for 30 minutes. The obtained 'buffy coat' (consistent of the peripheral blood mononuclear cells) was aspirated with a pipette and transferred into a new 50 ml Falcon tube, washed twice in 1 x PBS to extract the peripheral blood mononuclear cells (PBMCs) and then washed one more time in bead buffer.

Monocytes and/or $CD4^+$ T cells were extracted from the PBMC population by positive selection using incubation with either CD14 (for monocytes) or CD4 (for T cells) microbeads according to the manufacturer's instructions (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) whereby after the final wash, the cell pellet was re-suspended in bead buffer (80 µl for 10^7 cells) and 20 µl of the relevant microbead was added for every 10^7 cells. The suspension was kept on ice for 15 minutes after which the cells were washed again with PBS for 10 minutes. The bead-labeled monocytes or T cells were then separated from other PBMCs by usage of a magnetic-activated cell

separation column from Miltenyi Biotec. Cell viability was assessed using tryptan blue and was generally > 90%. Similarly, cell purity as determined by FACS analysis was > 95% in most cases (Fig 25).

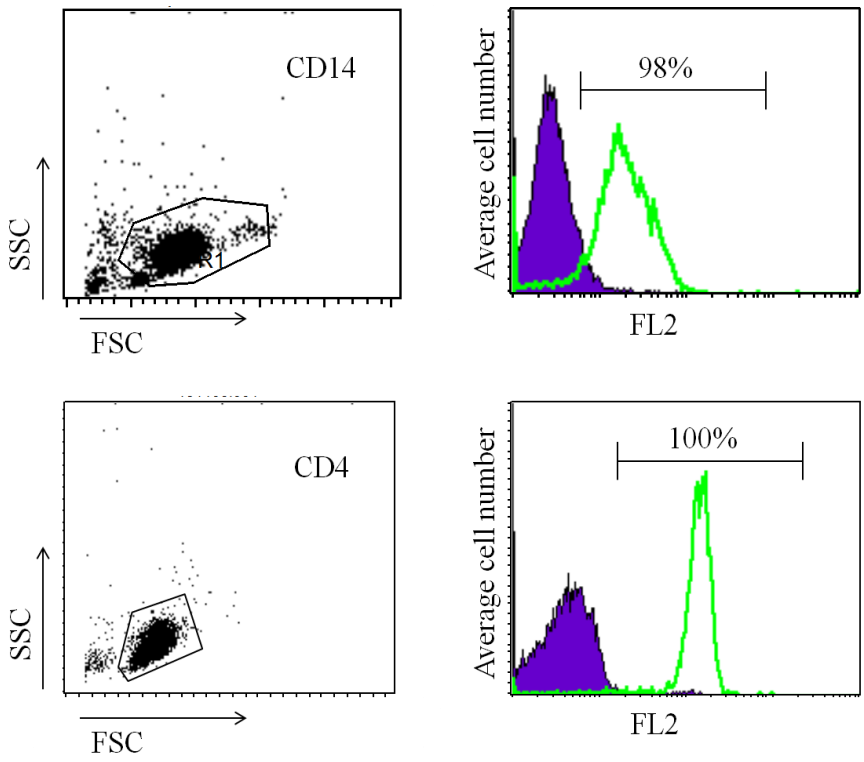


Figure 25. Representative purities of CD14+ and CD4+ cells using magnetic bead separation. The purple section on the left represents the cells stained with isotype control and the green section on the right, the $CD14^+$ (top panel) and $CD4^+$ (bottom panel) cells

8.2.2 Macrophage generation

To generate macrophages *in vitro*, PBMCs were first extracted as described above. After assessment of cell viability, cells were re-suspended in RPMI 1640 media with glutamine (but without FCS) and plated in 24-well tissue culture plates at a concentration of 10^6/ml, 1 ml/well. After 2 hours of incubation at 37°C and 5% CO_2, the wells were washed twice with warm PBS to remove non-attached cells. The remaining

attached monocytes were then cultured in macrophage media (0.5 ml/per well in 24-well plate). The media was replaced every 2 to 3 days with fresh media and matured macrophages were utilized at day 5-6 for subsequent experiments.

8.2.3 Monocyte and macrophage activation

To activate monocytes or macrophages, cells were purified and generated as described and cultured in the appropriate media in 24-well plates. C3a, C5a, LPS (100 ng/ml) and the combination of each anaphylatoxin with LPS were added to the culture and aliquots of cell supernatants and cell samples were collected at different time points for the subsequent analysis of cytokine production, surface receptor expression and caspase-1 activation. Various concentrations of C3a (5, 12.5, 25, 50 and 100 μM of the C3a agonist and 5, 12.5, 25, 50 and 100 nM of the serum purified C3a) and C5a (5, 12.5, 25, 50 and 100 nM) were used.

8.2.4 T cell activation

Isolated $CD4^+$ T cells were activated in flat-bottom 48-well culture plates (2.5-3 × 10^5 cells in 200 μl of media per well) which were coated with mAbs to CD3 and CD28 at 2.0 μg/ml each and incubated at 4°C overnight. Purified T cells were cultured and activated in the presence of 50 U/ml of recombinant human IL-2. T cell activation was then assessed by measurement of cell proliferation and cytokine production. For the specific *in vitro* induction of Th17 cells, recombinant IL-6 (25 ng/ml), IL-23 (25 ng/ml) and IL-1β (10 ng/ml) were added to the culture media as well as anti-IL-4 (10 μg/ml) and anti-INF-γ (10 μg/ml).

8.2.5 Assessment of cell surface receptor expression by fluorescence-activated cell sorting (FACS) analysis

The expression of C3aR and C5aR on monocytes and macrophages and of pannexin-1 channel on monocytes were assessed by FACS. Prior to incubation with relevant antibodies, monocytes and macrophages suspended in FACS buffer were incubated for 10 minutes in the refrigerator with Human Fc Receptor Blocking reagent (Miltenyi Biotec) according to the manufacturer's instructions (90 µl of FACS buffer plus 10 µl Fc Receptor Blocking reagent for 10^7 cells) to reduce unspecific antibody binding as these cell types express high amounts of Fc receptors. Washed and blocked cells were then incubated 20 min on ice with the appropriate amount of primary antibody (generally ranging from 0.2 µg/ml to 1µg/ml). In case the primary antibody was directly conjugated with a fluorochrome, the sample was then subjected to FACS analysis. As the primary pannexin-1 antibody was not fluorochrome conjugated, the Zenon PE Rabbit Conjugating Kit from Invitrogen was used based on manufacturer's instructions to label the antibody first prior to addition to cells. The labeling process involved addition of 1 µg of antibody to PBS with a final volume of ≤20 µL followed by addition of 5 µL of the Zenon rabbit IgG labeling reagent. The mix was kept in room temperature for 5 minutes before 5 µL of the Zenon blocking reagent was added to the mix. After 5 minutes of incubation in room temperature, the labeled antibody was ready to use.

Flow cytometeric analyses were performed using the Becton-Dickinson Fluorescence activated cell sorter (FACS Calibur) and data were analyzed using the CellQuest Pro software.

8.2.6 Measurement of caspase-1 activation

For assessment of Caspase-1 activation in monocytes, Green FLICA caspase-1 assay kit from Immunochemistry technologies (Minnesota, USA) was used according to the manufacturer's instructions. As described by the manufacturer, FLICA (Fluorescent Labeled Inhibitors of Caspases) probes are non cytotoxic and cell permeable. The probe covalently binds to active caspase in the cell and emits the green fluorescent signal while the unbound probe is removed during the wash step.

Monocytes stimulated with LPS, C3a or both for 2 hours, 4 hours and overnight (as well as un-stimulated monocytes) were prepared at a concentration of $5x10^5$/ml and 290 µl of each was placed in eppendorf tubes. Cells were incubated with 10 µl of FLICA stain at 37^oC for 1 hour. They were then washed twice with wash buffer provided in the kit and suspended in FACS buffer and analyzed with flow cytometry.

8.2.7 ATP measurement

ENLITEN ATP assay system from Promega was used to measure ATP in cell culture supernatants using manufacturer's instructions.

Monocytes were stimulated overnight with LPS with and without C3aR agonist. The supernatants were removed the next day and centrifuged to remove cells and debris. 100 µl of diluted rL/L reagent from the kit was added to 100 µl of supernatant from each cell culture condition and the light output from the reaction of the rL/L reagent with the ATP in supernatants was measured by luminometer (Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany). Results were compared with the standard curve which was generated using serial dilutions of standard (ATP) available in the kit, addition of rL/L and measuring the light output with the luminometer.

8.2.8 Measurement of cytokine production

8.2.8.1 A. Enyme-Linked Immunosorbent Assay (ELISA)

At the time point of interest, cell supernatants were collected from the culture plates and centrifuged at 1000 rpm to clear off cell debris. The supernatants were either used immediately or stored in -40^{o}C until use.

Ready-Set-Go! ELISA kits and Corning Costar ELISA plates from eBiosciences (Hatfield, UK) were used for all ELISA experiments according to the manufacturer's instructions. Briefly, ELISA plates were coated with 100 µl/well of capture antibody in Coating Buffer (1:250) and incubated overnight at 4°C. Plates were washed 5 times with ELISA wash buffer and dried on absorbent paper to remove residual buffer. Wells were blocked with 200 µl/well of Assay Diluent for 1 hour at room temperature. The plates were washed and dried as before and 100 µl of the standard solution and samples were added in duplicates. For the standard curve, the standard was prepared in 2-fold serial dilutions (in assay diluent) from the maximum concentration that was recommended by the manufacturer to a sample without standard addition. Cell culture supernatants were generally diluted 1:1 in diluent buffer (for monocyte IL-1β the concentration was reduced to 1:10 due to high amounts of the cytokine). The plates were incubated at room temperature for 2 hours after which they were washed 5 times. 100 µl of detection antibody (1 in 250 dilution in the assay diluent) was added per well and incubated in room temperature for 1 hour. After washing the plate as before, 100 µl/well of Avidin-HRP diluted in assay diluent (1:250) was added and the plate was incubated at room temperature for 30 minutes. Following 5 more washes, 100 µl of TMB (Tetramethylbenzidine) substrate solution was added to each well and the plate was incubated in room temperature for 15 minutes after which 50 µl of 1M sulphuric

acid (H_2SO_4) was added to each well to terminate the reaction. Optical density was measured on an ELISA plate reader (Specramax Plus, Molecular Devices) at 450 nm. The concentration of each cytokine was calculated in comparison with the standard curve made from optical densities of the known concentrations of the standard.

8.2.8.2 B. Intracellular cytokine staining

Purified and cultured cells were re-stimulated with a combination of 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and 500 ng/ml ionomycin (Sigma) for 4 hours. 3 µM Monensin (Biolegend, 420701) was added for the last 2 hours of the culture to induce accumulation of cytokines in the golgi apparatus of the cell. The cells were then harvested and washed twice in 1x PBS. Fix and Perm reagent from Invitrogen (GAS004) was added at 100 µl to each sample and incubated for 15 minutes at room temperature. The cells were washed with 1 ml of FACS wash buffer and centrifuged for 5 minutes at 1400 rpm. The supernatant was aspirated and the cell pellet re-suspended with a gentle vortex. 100 µl of permeabilization medium containing an appropriate amount of the desired antibody (generally 0.2 µg/ml to 1 µg/ml) or the corresponding isotype control was added, vortexed for 1-2 seconds and incubated on ice for 20 minutes. The cells were washed again using 1 ml of wash buffer and centrifuged for 5 minutes at 1400 rpm and re-suspended in 250 µl of the wash buffer and analyzed by flow cytometry.

8.2.9 Histology

The rejecting and non-rejecting renal transplant biopsy samples were obtained from Professor Terrence Cook at Imperial College (London) and were from patients who had given consent to use of biopsy material surplus to diagnostic requirements.

Hematoxylin-Eosin staining: Formalin-fixed, paraffin-embedded tissue samples were de-paraffinized by incubation in a 60ºC-heated oven for 1 hour after which they were immersed in xylene for 10 minutes followed by hydration using decreasing concentrations of ethanol (slides were put in 100%, 90% and 70% ethanol for 5 min each). After washing the slides with PBS, they were incubated with hematoxylin for 10 minutes, washed under running water and incubated with eosin for 30 seconds after which they were washed again under running water and dehydrated through increasing concentration of alcohol (put in 70%, 90% and 100% ethanol for 5 min each) followed by immersion in xylene for 10 minutes and then mounted in permount.

Immunohistochemistry: Formalin-fixed, paraffin-embedded samples were de-paraffinized and hydrated through decreasing concentrations of ethanol as described above. Heat-induced antigen retrieval was conducted in 10 mM sodium citrate solution (pH=6.0) for 30 minutes. Slides were washed three times in PBS, and incubated with 5% BSA for 60 minutes at room temperature to block non-specific binding. Primary antibodies (anti-CD4, anti-IL-17, and anti-C3a) were diluted to manufacturers' recommended concentrations and applied to each sample and incubated at 4°C overnight. Secondary antibody staining was conducted for 60 minutes at room temperature. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were taken using the Nikon A1R Si confocal microscope. Dr Hide Yamamoto, a postdoctoral fellow in our laboratory helped me with the staining and together with Dr John Harris from the NIKON Imaging Centre at KCL (King's College London) assisted with obtaining the confocal microscopy images.

8.2.10 Statistical analyses

Unpaired Student t-test was used to compare groups (with Graphpad software) and data were presented as mean ± standard deviation. $P < 0.05$ was defined as significant.

8.3 Results

Similar to the injury-inducing role of complement activation products, several studies have indicated that elevated levels of the pro-inflammatory cytokine IL-1β is associated with kidney IRI (Furuichi et al., 2006, Rusai et al., 2008). It has also been shown that IL-1β production is the major and most important end product of inflammasome activation (Franchi et al., 2009). Based on these observations and the fact that some complement components (anaphylatoxins in particular) play an important role in kidney IRI, which I have discussed in detail in the first chapter of my thesis, I hypothesized that complement has a role in activation of inflammasome and production of IL-1β in kidney IRI.

Knowing that monocytes and macrophages are the main sources of IL-1β production, I planned to first assess if stimulation of these cells with anaphylatoxins with or without TLR4 stimulation (via LPS addition) leads to production of IL-1β. Although production of IL-1β by monocytes with anaphylatoxin stimulation has been reported before (Haeffner-Cavaillon et al., 1987), there was controversy over contamination of the anaphylatoxin used with endotoxins (Arend et al., 1989).

8.3.1 Expression of C3a and C5a receptors by monocytes and macrophages

The first set of experiments in this part of my thesis was aimed at investigating if stimulation of monocytes and macrophages with anaphylatoxins C3a or C5a leads to production of the key effector cytokine IL-1β by these cells. To assess if these cells are

able to respond to the complement activation fragments, C3aR and C5aR expression on freshly isolated monocytes and on M-CSF-differentiated macrophages were first measured (5 days after their culture) by FACS analysis.

Up to 10^7 freshly isolated human monocytes and M-CSF-differentiated macrophages were initially suspended in 90 µl of FACS buffer and incubated with 10 µl of Fc receptor block (miltenyi) to reduce nonspecific binding of antibody, then washed with PBS and incubated with PE-conjugated human anti-C3aR antibody, FITC-conjugated anti-C5aR antibody or their respective isotype control antibodies (5 µl of antibody was used for an average of 10^6 cells suspended in 100 µl of FACS buffer). Figure 26a demonstrates that freshly isolated monocytes express both C3a and C5a receptors in ample amounts. In contrast, the expression pattern of these receptors changes visibly in monocyte-derived macrophages (Figure 26b): although all cells express comparable amounts of the C5aR, the expression of C3aR on macrophages was only about 50% of cells compared to monocytes. Nonetheless, these data show that monocytes and macrophages express anaphylatoxin receptors and are therefore equipped to respond to C3a (in the case of macrophages about half of the cells) and C5a.

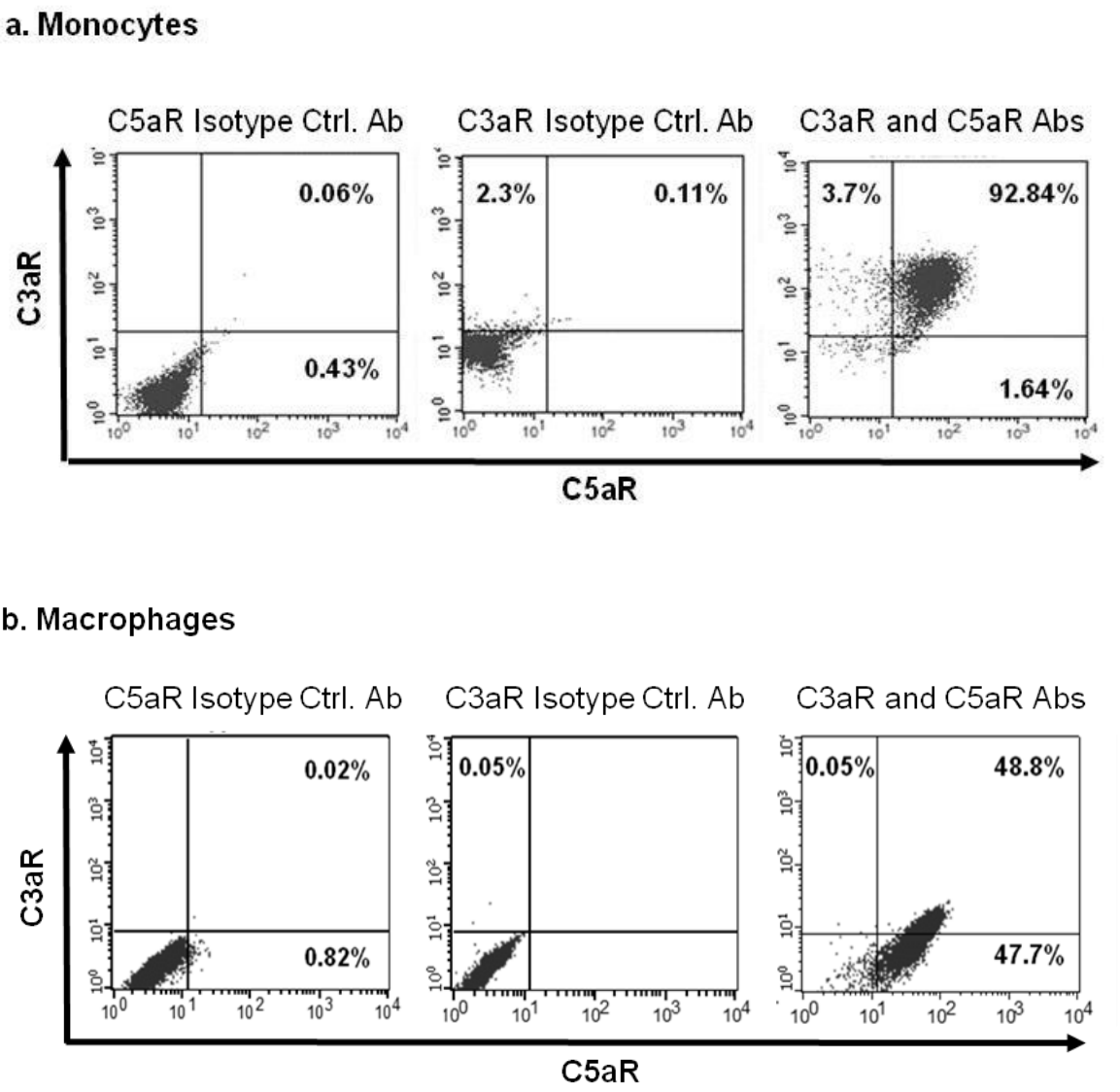


Figure 26. Monocytes and macrophages express C3a and C5a receptors. C3aR and C5aR expression was assessed on freshly isolated human monocytes (a) or on 5d M-CSF-differentiated macrophages (b) by FACS analysis. Shown is one representative of three similarly performed experiments using a different donor each time.

8.3.2 Impact of C3a and C5a on monocyte and macrophage IL-1β production

8.3.2.1 Monocytes

To investigate if anaphylatoxins C3a and C5a affect IL-1β production by monocytes, fresh blood was obtained from volunteer donors and monocytes were isolated using CD14 beads as described in section 8.2.1. To overcome the risk of contamination with

endotoxins, a C3aR agonist (generated under endotoxin-free conditions) was used in these experiments. However, for C5aR stimulation experiments, I had to use recombinant C5a as there is no chemical C5aR agonist available. C3aR agonist had similar effects compared with purified C3a (Figure 29). Cells were stimulated with different doses of the C3aR agonist or recombinant C5a with and without 100 ng/ml of LPS. LPS was used in this model as its presence as a TLR4 agonist has been shown in previous studies to be a stimulator for IL-1β production in monocytes and macrophages (Beutler, 2000, Chow et al., 1999). Furthermore, TLR4 has been shown to have a role in tissue injury in both native and transplant models of kidney IR (Kruger et al., 2009, Pulskens et al., 2008). Doses of both C3a and C5a were titrated: 25 μM of C3aR agonist (equivalent to 100 nM of purified C3a) and 50 nM of recombinant C5a consistently induced the most effect (for IL-1β production); therefore these doses were used in future experiments. A wide range of anaphylatoxin concentrations (from 10-300 nM for C3a and 5-100 nM for C5a) have been used on different cell types in other studies and the dose I used represents an average (Hartmann et al., 1997, Schraufstatter et al., 2009, Monsinjon et al., 2003). The cells were incubated at 37°C in 5% CO_2 and cell supernatants were removed at different time points of 1, 2 and 4 hours as well as from the overnight incubated samples for measurement of IL-1β, IL-6 and IL-23 by ELISA. Since the overnight stimulated samples showed the highest amount of cytokine, this time point was selected for all cytokine production experiments.

As shown in Figure 27, LPS induced IL-1β production from monocytes, as expected from previous studies (Netea et al., 2009). Addition of C3aR agonist to LPS-treated monocytes increased their IL-1β production up to 4-fold after overnight incubation in a dose dependent manner (Figure 29). Interestingly, addition of the C3aR agonist alone to the monocyte culture had no effect. This observation is in contrast to earlier findings

where serum-purified C3a (in comparable doses used in my experiment) and C3adesArg alone induced IL-1β from PBMCs (Haeffner-Cavaillon et al., 1987). I observed that recombinant C5a does not affect IL-1β production in this system. However, C5a was active and modulated monocyte function as it was detected – in line with previous publications (Hashimoto et al., 2010) – to strongly induce IL-6 in an LPS-independent fashion (Figure 27b). Neither C3a nor C5a had an effect on the production of IL-23, a cytokine produced by activated monocytes and by DCs, vital in the induction of Th17 effector responses (Fig 27c).

Thus, these data demonstrate that C3aR engagement significantly and specifically increases LPS-mediated IL-1β production by human monocytes.

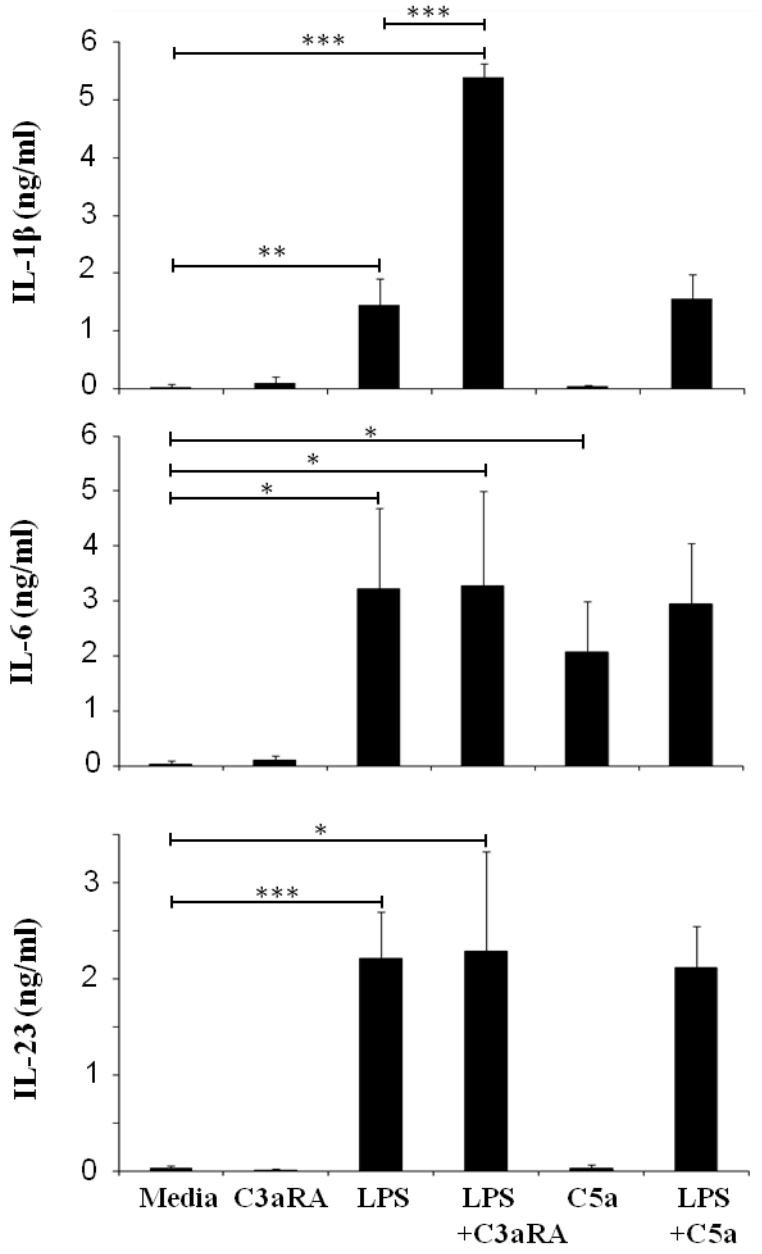


Figure 27. C3a increases LPS-mediated IL-1β production by human monocytes. Cytokine production by monocytes after LPS and anaphylatoxin activation. Freshly isolated monocytes were incubated overnight in media or in media with LPS (100 ng/ml) with and without addition of human C3a receptor agonist (25 μM) or recombinant human C5a (50 nM). IL-1β, IL-6 and IL-23 levels were measured in supernatants by ELISA. *, $p < 0.05$; **, $p < 0 .005$; ***, $p < 0.001$.

8.3.2.2 Macrophages

When monocytes encounter activating signals, they produce pro-inflammatory cytokines and can also further develop into DCs or macrophages, depending on the

environmental cues they encounter. Noting the pathological influence of macrophages as well as anaphylatoxins in IRI and kidney transplant rejection (Chadban et al., 2010; Li and Okusa, 2010), I wanted to next determine if C3a and/or C5a is also able to modulate cytokine production of macrophages and possibly participate in tissue injury. Thus, production of several key cytokines involved in tissue inflammation and immune cell activation such as IL-1β, IL-6 and IL-23 were measured from macrophages after stimulation with C3a, C5a and LPS or their combination.

Monocyte derived macrophages were prepared as described in section 8.2.2. Initially, expression of C3a and C5a receptors on these cells were confirmed (Fig 26b). After 5 days of culture, the cells were stimulated (in duplicate wells) overnight with 25 μM C3a agonist, 50 nM recombinant C5a, 100 ng/ml LPS or combination of LPS with either anaphylatoxin. The supernatants were removed, centrifuged at 300 rpm and kept in -40°C until used.

These experiments resulted in some key observations (Figure 28). First, anaphylatoxin and LPS-stimulated macrophages produced substantially lower cytokine levels compared to monocytes (about 10-15 times less in the case of IL-1β and IL-23 and half the level of IL-6). Second, C5a was able to positively regulate IL-6 secretion in macrophages, as with monocytes but even more so. Lastly and most importantly, IL-1β production was critically dependent on addition of both C3aR agonist and LPS, as only cells treated with both agents generated IL-1β.

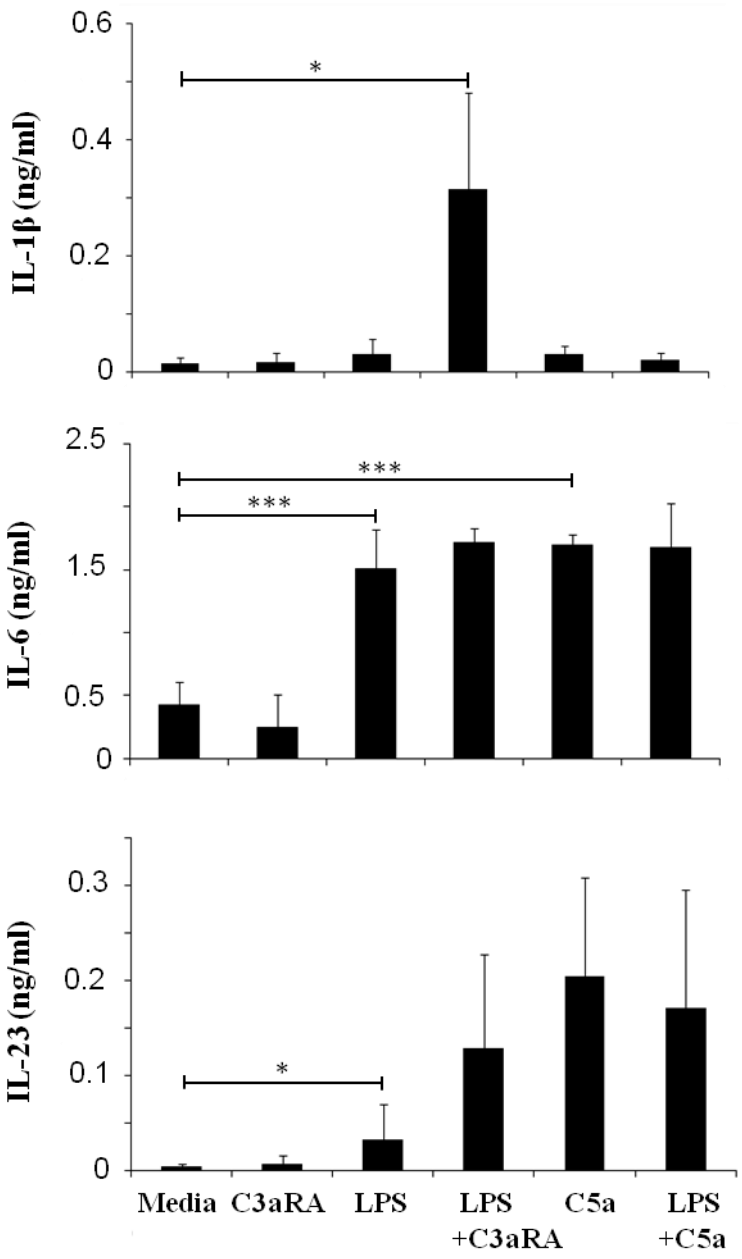


Figure 28. IL-1β production by macrophages requires both LPS and C3a. Freshly purified human monocytes were differentiated into macrophages by M-CSF incubation for 5 days. Macrophages were then incubated overnight in the presence of LPS (100 ng/ml) with or without addition of either C3aR agonist (25 μM) or recombinant C5a (50 nM). IL-1β (top panel), IL-6 (middle panel) and IL-23 (lower panel) levels in the cell supernatants were measured by ELISA. Data represent mean ± SD (n=3). *, $p < 0.05$; ***, $p < 0.001$.

These data indicate that there are substantial differences in regards to cytokine production between monocytes and macrophages in the response towards anaphylatoxins. The lower level of cytokine production in macrophages in response to

C3a in comparison to monocytes can be in part related to lower C3aR expression on these cells (shown in Figure 26b). This phenomenon could be a reflection of the different physiological roles of monocytes and macrophages in host defense. It could be that monocytes being the first line of defense in the blood stream, are more sensitive to certain stimuli and are able to initiate a more potent immune response, whereas macrophages which mostly reside in tissues need to have tight control in their response so that they don't damage the surrounding tissue – or even further, switch into a tissue repair programme. Difference in processing intracellular pro-IL-1β due to different expression of ion exchange channels and caspase-1 activation between monocytes and macrophages has been suggested to play a role in variation of IL-1β release between the two cell groups (Perregaux et al., 1996, Kahlenberg and Dubyak, 2004).

Considering the less robust production of IL-1β by C3a stimulated macrophages compared to monocytes, I concentrated on monocytes in future experiments.

8.3.3 C3aR agonist and pooled human C3a have comparable effects on IL-1β production by human monocytes

C3aR agonist was chosen to initiate these experiments rather than purified C3a. The reasoning behind this was that earlier work had shown LPS-independent induction of IL-1β with purified C3a in monocytes (Haeffner-Cavaillon et al., 1987) but the criticism in these experiments was that it could never be convincingly excluded whether the C3a preparation was LPS-free (Arend et al., 1989). The use of a chemical compound would potentially exclude LPS contamination (although contamination during the purification process is still a possibility). My finding that the C3aR agonist alone does not induce IL-1β in monocytes strongly suggests that a TLR-mediated second signal is indeed

required for IL-1β induction. A reliable agonist for the human C5aR is currently not available.

Thus, to investigate if C3a, purified and pooled from human serum, has a similar effect as the C3aR agonist, increasing doses of either purified C3a or the C3aR agonist were added to monocyte cultures together with LPS (Figure 29). Addition of C3aR agonist within a range of 25 to 50 µM or purified C3a in 50 to 100 nM were found to produce comparable results. Purified C3a in the absence of LPS did not induce IL-1β. Interestingly, when the C3aR agonist or purified C3a were used in high doses (125 µM and 500 nM, respectively), both had an inhibitory effect on LPS-induced IL-1β production. The reason for this is unclear, but may be due to reduced expression of the anaphylatoxin receptors that is observed particularly when G protein-coupled receptors are activated with high doses of their ligands (Li et al., 2012). Stimulation of the G protein-coupled receptors by their ligands has been shown to result in their internalization leading to desensitization to the ligand (Pierce et al., 2002). This could be a self regulatory mechanism which prevents over-activation of the immune system in response to large amounts of inflammatory stimuli.

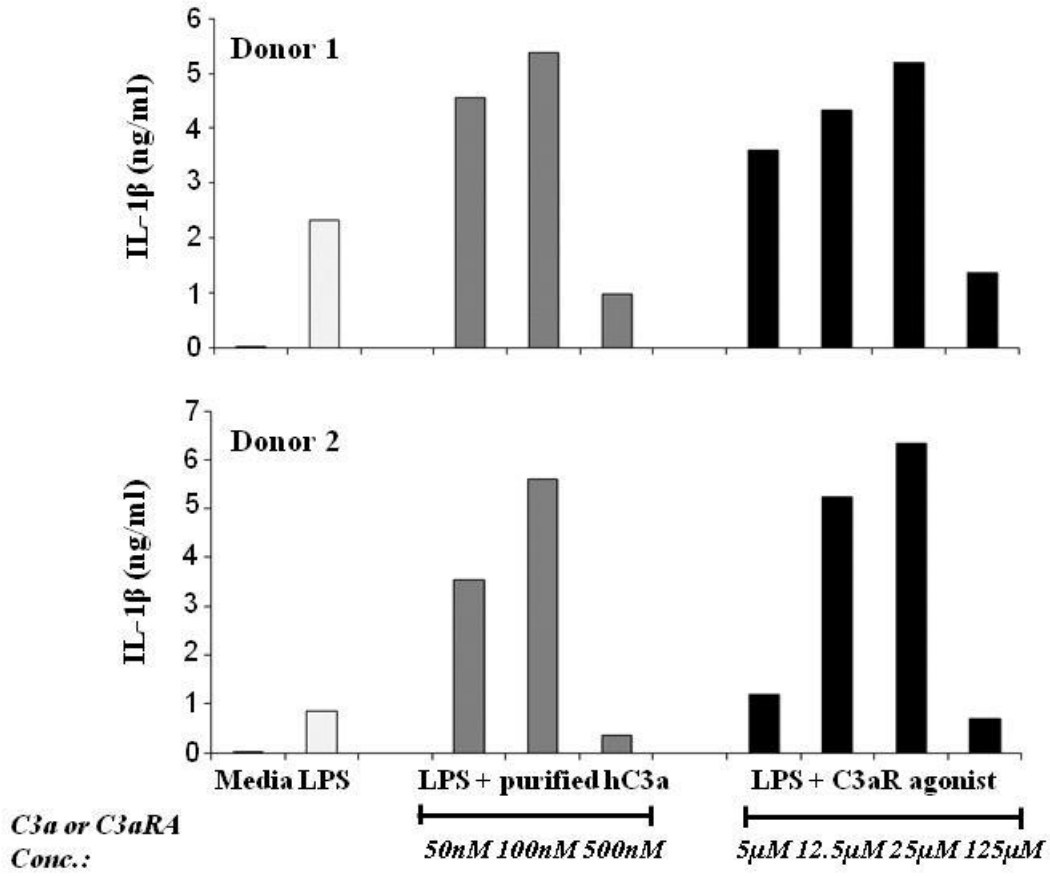


Figure 29. Serum-purified C3a and C3aR agonist have comparable effects on IL-1β production by human monocytes. Freshly purified monocytes from two different donors were left untreated (media) or activated with LPS (100 ng/ml) and increasing amounts of either serum-purified pooled C3a or C3a receptor agonist (C3aRA) and IL-1β production was measured after overnight activation. Bars represent the median values of each condition performed in triplicate.

Because the C3aR agonist is cheaper to use than human purified C3a and its use avoids batch variation, C3aR agonist was chosen (at a dosage of 25 μM) throughout the remainder of the study.

8.3.4 C3a-exposed monocytes induce strong Th17 responses

Knowing that IL-1β is a vital requirement for human Th17 induction (Acosta-Rodriguez et al., 2007), I wanted to address the potential functional importance of the observation that C3a is an inducer of IL-1β production in human monocytes. My hypothesis was that C3aR agonist-treated monocytes (with or without TLR4 stimulation with LPS) may

be prime inducers of Th17 responses. To test this hypothesis, $CD4^+$ T cells were isolated from freshly obtained blood using CD4 beads and cultured in plates pre-coated with CD3 and CD28 as described in sections 8.2.1 and 8.2.4 respectively. 400 µl of culture media was used for negative control T cells. To induce the differentiation of Th17 cells for use as positive control, recombinant IL-6 (25 ng/ml), IL-23 (25 ng/ml) and IL-1β (10 ng/ml) were added to the culture media as well as anti-IL-4 (10 µg/ml) and anti-INF-γ (10 µg/ml) (to prevent the development of Th1 or Th2 cells). 200 µl of monocyte supernatant after overnight stimulation with 25 µM C3a agonist, 100 ng/ml of LPS and their combination was added to 200 µl of media in the experiment group of $CD4^+$ T cell cultures. After 3 days, the cells were stained for intracellular IL-17 and analyzed by FACS. The results shown in Fig 30 demonstrate that supernatant from the monocytes activated with LPS and C3a were the strongest inducer of IL-17 production where the intracellular IL-17 was almost twice as much as the amount detected in Th17-skewed T cells. LPS-only stimulated monocyte supernatant also increased the IL-17 production of T cells 1.3 times compared to Th17 skewed T cells. The increased production of IL-17 in these conditions suggests that the $CD4^+$ T cells exposed to supernatant of monocytes stimulated with LPS and more so with LPS and C3a have developed a Th17 phenotype. Supernatants from non-stimulated monocytes and C3a-only stimulated monocytes had an effect in increasing IL-17 production compared with non-skewed T cells (about 1.5 times) but not compared with Th17 skewed T cells (Figure 30).

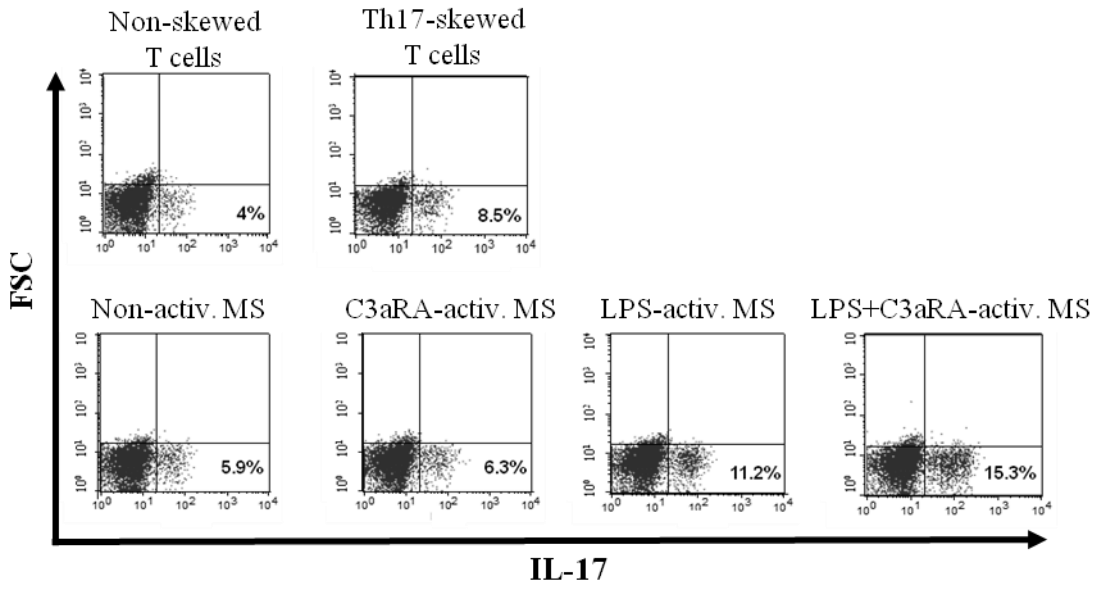


Figure 30. C3a-exposed monocytes induce strong Th17 responses. Human $CD4^+$ T cells respond to supernatants from LPS + C3a-activated monocytes with increased IL-17 secretion. Freshly purified human $CD4^+$ T cells were activated with immobilized antibodies to CD3 and CD28 in the presence of media only (non-skewed T cells, negative control) or media containing rIL-6, rIL-23 and rIL-1β (Th17-skewed T cells, positive control). To assess the effect of C3a-mediated cytokine production by monocytes in this system, T cells were activated with 1:1 mixtures of supernatants from monocyte cultures (MS) that had not been activated or activated with C3a alone, LPS or LPS and C3a. Th17 induction was measured 3 days post activation by intracellular cytokine staining. Shown is one representative of three similarly performed experiments.

To investigate how much of the Th17 induction in the above experiment is dependent on IL-1β production by monocytes, the effect of IL-1β inhibition using IL-1Ra in the $CD4^+$ T cell culture prior to addition of monocyte supernatants was assessed. Results depicted in Figure 31 shows that IL-17 production was inhibited to a large extent following the addition of IL-1Ra. However, of note, IL-17 induction was only partially reduced by this treatment. This could suggest that IL-1β inhibition by IL-1Ra is incomplete or that additional Th17-driving factors (for example IL-6) which are secreted by LPS as well as LPS and C3aR agonist-treated human monocytes can continue to drive Th17 differentiation to some extent.

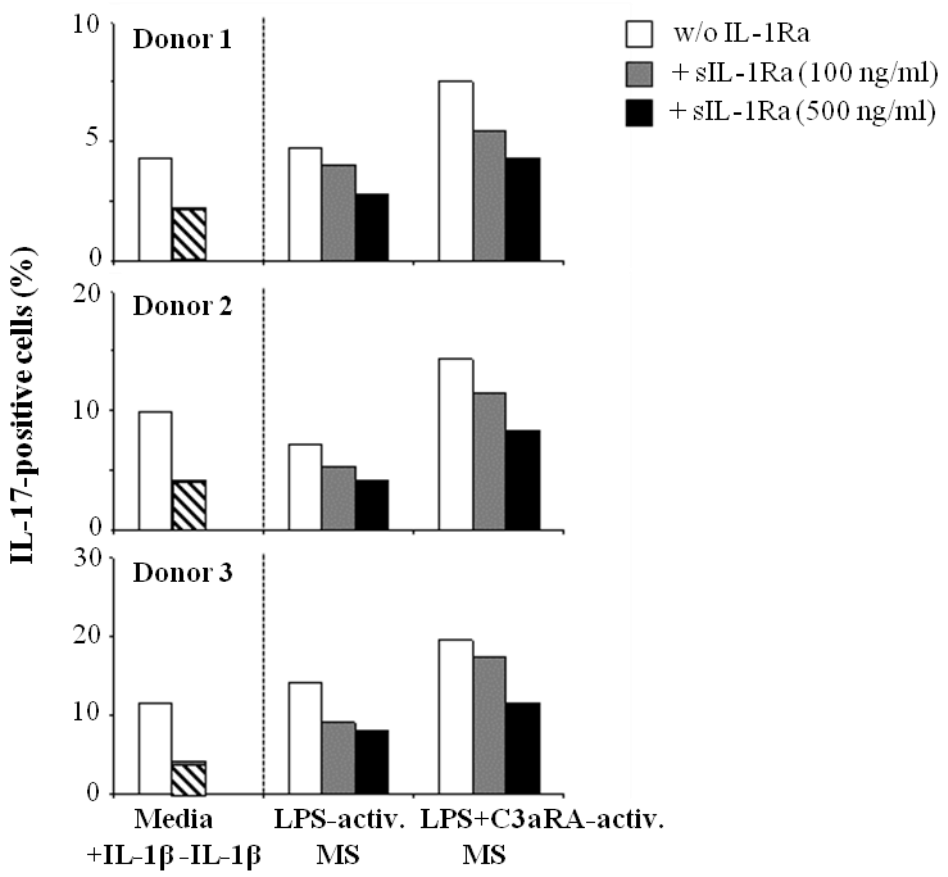


Figure 31. Functional inhibition of C3a-induced monocyte-derived IL-1β significantly decreases Th17 induction. Monocyte supernatants (MS) from LPS and LPS/C3a stimulated cells were used in the absence or presence of increasing amounts of an IL-1 receptor antagonist (IL-1Ra) and IL-17 production was measured by intracellular staining and assessment by FACS. Bars represent the mean values of each condition performed in duplicate with cells from three different donors.

These findings suggest that LPS/C3a-activated monocytes can drive strong Th17 induction (15% IL-17$^+$ T cells in *in vitro* cultures indicates excellent Th17 induction as no current *in vitro* induction condition leads to more than 20% IL-17$^+$ cells) and that one key cytokine involved is indeed IL-1β (likely beside IL-6, driven by C5a and IL-23 produced upon LPS exposure).

8.3.5 Rejecting human kidney tissue contains areas of high C3a and Th17 presence

After demonstrating that LPS and C3a-treated human monocytes produce high amounts of IL-1β and can induce strong Th17 responses in human T cells, validating the core of my hypothesis, the relevance of this phenomenon in kidney transplant rejection was investigated. Biopsy samples of kidney transplant tissue from patients with acute cellular rejection as well as non-rejecting kidney transplant tissue were stained for CD4, C3a and IL-17 as described in section 8.2.9. Notably, only tissue from rejecting but not non-rejecting kidneys contained large areas of C3a and Th17 accumulation (Figure 32).

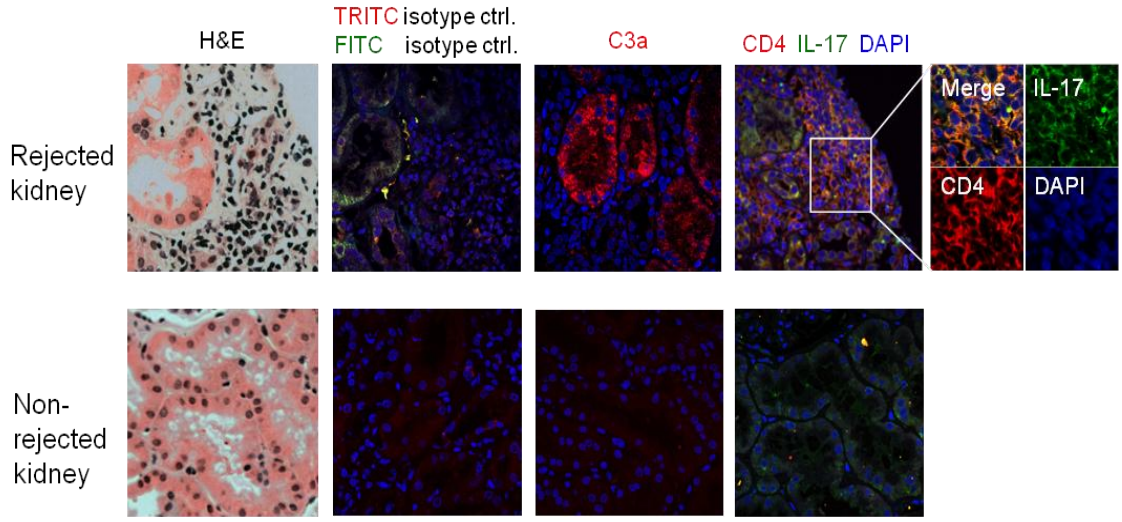


Figure 32. Rejecting human kidney tissue contains areas of high C3a and Th17 presence. Confocal microscopy following immunohistochemical staining for CD4, C3a, and IL-17 on kidney transplant biopsy samples with acute cellular rejection (upper panel) and no rejection (lower panel). Infiltration of CD4 cells as well as presence of C3a and IL-17 is seen only in the rejected sample. Results shown are representative of histology data obtained from samples of three patients with acute transplant kidney rejection and two patients without signs of kidney rejection (60x magnification).

A more detailed view of the rejected kidney tissue demonstrated the presence of both $CD4^+$ cells and IL-17 producing cells close to the tubular epithelial cells (Figure 33A). Comparing $IL\text{-}17^+$ $CD4^+$ vs $IL\text{-}17^-$ $CD4^+$ cells in a minimum of four fields per sample,

about one quarter of the $CD4^+$ T cells were found to be IL-17^+. H&E staining of kidney transplant tissues followed by staining with anti-CD68 antibody demonstrated co-localization of T cells with $CD68^+$ cells (monocyte/macrophages) in the rejecting samples (Figure 33B).

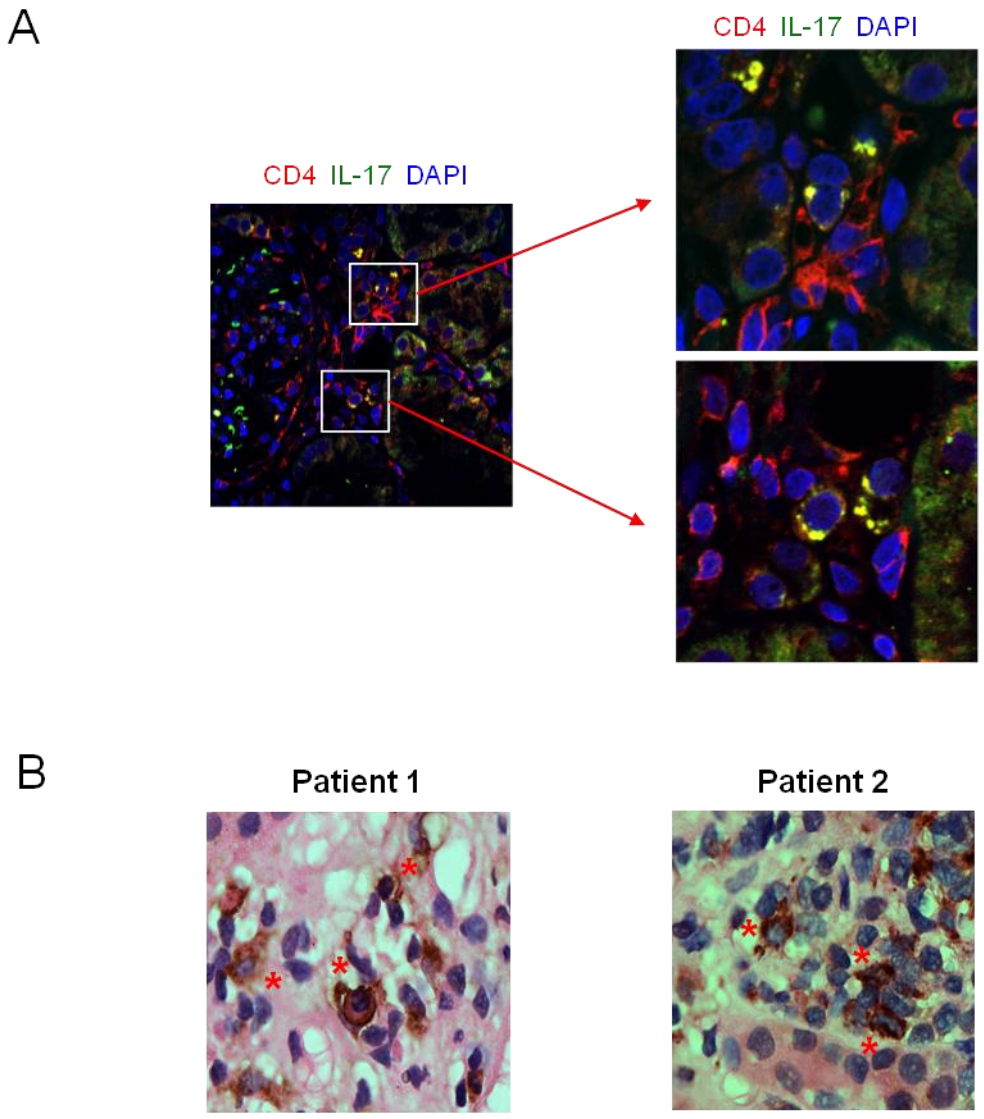


Figure 33. Acutely rejecting transplant kidney grafts are infiltrated with macrophages and Th17 cells. (A) Confocal fluorescence microscopy following immunohistochemical staining for CD4 and IL-17 on kidney transplant biopsy samples with acute cellular rejection. This is a representative slide from 3 patient samples analyzed. 20-25% of infiltrating T cells were Th17 cells. This was calculated by comparing IL-17^+ $CD4^+$ vs. IL-17^- $CD4^+$ cells in a minimum of four fields per sample. Left panel, 60x magnification; right panels, 100x magnification. (B) Staining of 2 rejecting kidney biopsy samples with H&E and anti-CD68 followed by DAB visualization demonstrates the co-localization of T cells with $CD68^+$ cells (representative of monocytes/macrophages). 100x magnification; *, denotes macrophages.

The above results suggest that production of C3a as well as DAMPs from the injured tubules in the rejecting kidney stimulate the infiltrated monocytes/macrophages, resulting in their production of the inflammatory cytokines that facilitate the differentiation of $CD4^+$ T cells to IL-17 producing cells, potentially leading to more tissue damage. However, I would like to stress that the stainings indeed support this model but do not deliver unequivocal evidence. For this, likely an *in vivo* transplantation study using mice deficient in monocytes should be performed.

8.3.6 ATP regulates the C3a-mediated increase in monocyte IL-1β production

My data so far suggest that complement C3 activation contributes to monocyte-driven Th17 induction and that acutely rejected kidney tissues contain the 'triangle of factors' required for local detrimental Th17 generation in IR: C3a, monocyte-like/derived cells as well as Th17 T cells all in close proximity.

To further understand which intracellular signals following C3aR activation may contribute to IL-1β secretion, several potential pathways were considered. As ATP-release from platelets following C3a stimulation had previously been reported (Collard et al., 2000a) and ATP had been shown to be a stimulus for IL-1β secretion from monocytes (Perregaux et al., 2000), a functional relationship between C3a, ATP and IL-1β production in monocytes was assessed.

To examine the role of ATP in the C3a-mediated increase in IL-1β production in monocytes, 300 µM oxidized ATP (oxATP) was used in monocyte cultures 15 minutes prior to addition of C3a with or without LPS. oxATP, which is widely used as a selective inhibitor of P2X7 receptor signaling, induces irreversible blockade of the extracellular ATP effect on cells (Murgia et al., 1993).

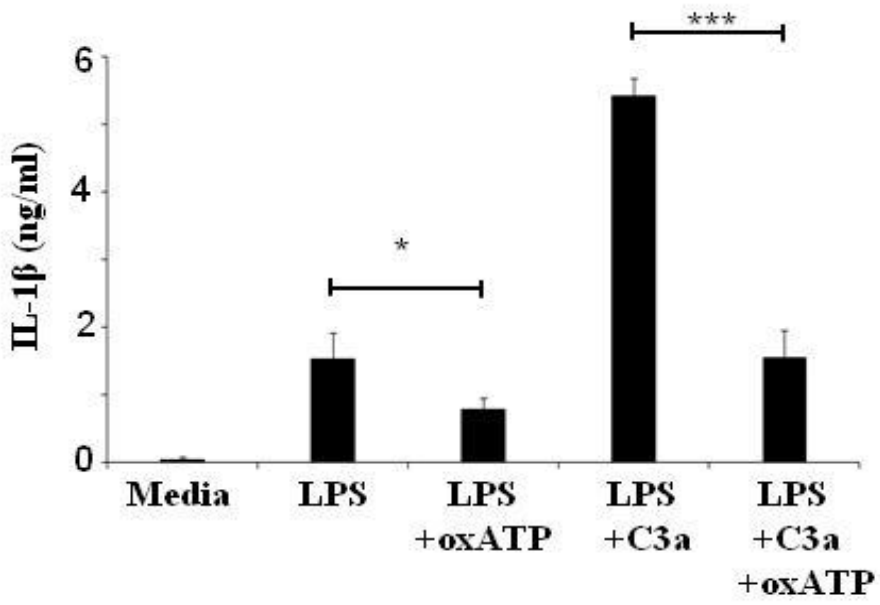


Figure 34. C3a-mediated increase in monocytic IL-1β production is modulated by ATP. Monocyte treatment with oxidized ATP abrogates LPS/C3a-mediated IL-1β production. Freshly purified monocytes were either left untreated or treated for 15 minutes with 300 μM oxidized ATP (oxATP) before overnight activation with LPS (100 ng/ml) and C3aR agonist (25 μM) and subsequent assessment of IL-1β secretion. Data represent mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$

The significant reduction of IL-1β production by monocytes stimulated with LPS and C3a after blocking of the ATP effect indicated that ATP is an important mediator of C3a-induced increase in IL-1β (Figure 34).

8.3.7 Pannexin-1 mediates the release of ATP from monocytes

Considering that Panx1 is one of the cellular channels able to mediate the release of ATP to the extracellular environment (Locovei et al., 2006a), it was plausible that it has a role in ATP release from monocytes as well. To test this hypothesis, the expression of Panx1 on resting and on LPS- or LPS+C3aR-activated monocytes was initially assessed.

Monocytes were activated overnight with C3aR agonist with and without LPS. Anti-Panx1 antibody was conjugated with phycoerythrin dye using a kit from Invitrogen as described in section 8.2.5. After incubation of monocytes with Fc block, they were then incubated with the conjugated anti-Panx1 antibody and assessed by flow cytometry.

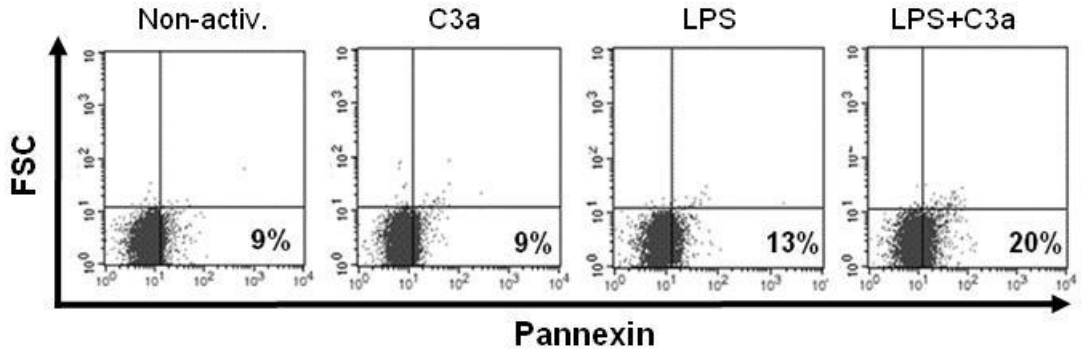


Figure 35. C3aR activation increases cell surface expression of pannexin-1 channel. Monocytes were activated as indicated and pannexin-1 channel expression on the cell surface was measured by FACS analysis 4 hours post activation. Shown is one representative of three similarly performed experiments.

As demonstrated in Figure 35, resting monocytes express Panx1 albeit in relatively low amounts. In line with the previous data sets, addition of C3aR agonist alone did not induce changes to Panx1 expression, while LPS stimulation resulted in moderate increase in Panx1 expression on monocytes. Co-stimulation with C3aR agonist and LPS increased this expression to more than double the amount as compared to non-stimulated cells.

To further assess if Panx1 and C3aR-mediated Panx1 up-regulation is responsible for release of ATP to the extracellular space in monocytes, 200 µM carbenoxolone, an agent frequently used for blocking gap junctions and with a known Panx1 inhibitory activity (Bruzzone et al., 2005), was used in overnight LPS and C3a stimulated

monocyte cultures as a Panx1 blocking agent (carbenoxolone was added 15 minutes prior to stimulating the cells with C3a and LPS).

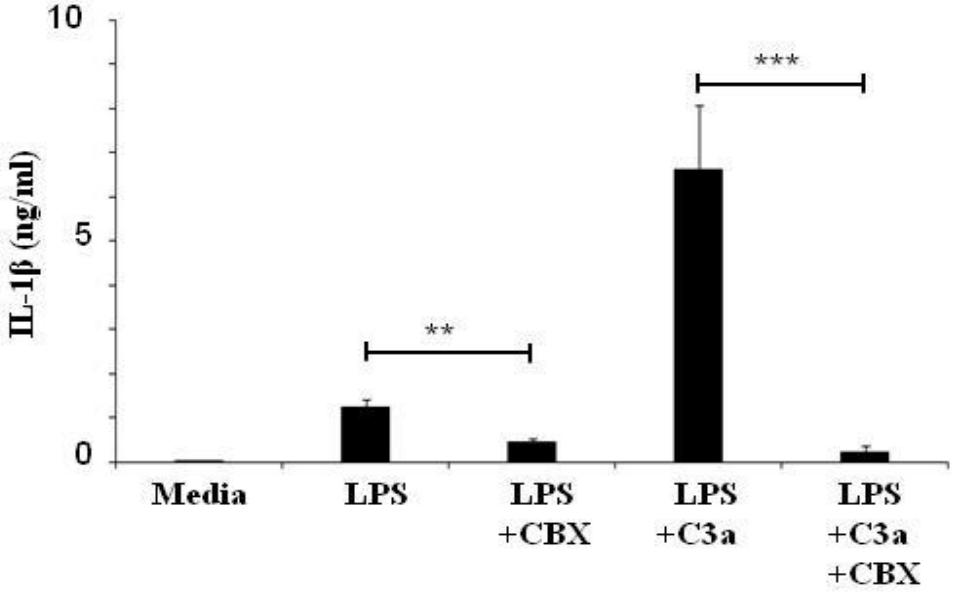


Figure 36. Inhibition of Panx1 channel by carbenoxolone impairs LPS/C3a-mediated IL-1β secretion. Freshly purified monocytes were either left untreated or treated with 200 μM carbenoxolone (CBX) 15 minutes before overnight activation with LPS (100 ng/ml) with or without C3aR agonist (25 μM). Supernatants were subsequently assessed for IL-1β with ELISA. Data represent mean ± SD (n=3). **, p < 0 .005; ***, p < 0.001

The marked inhibition of IL-1β production following blockage of Panx1 channel with carbenoxolone (Figure 36) in addition to the similar effect with inhibition of ATP indicates that Panx1 is responsible for release of ATP in LPS and LPS/C3a-stimulated monocytes which leads to IL-1β production.

Controlled ATP release by living cells via Pannexin channels is a means for immunomodulation (Schenk et al., 2008, Woehrle et al., 2010). However, ATP can also be released into the environment by uncontrolled mechanisms, such as by dying or injured cells through their compromised membranes (Rock and Kono, 2008). To

exclude that ATP release from monocytes following LPS and C3a stimulation is due to increased death in these cells, monocyte cultures were assessed via FACS after one day in culture with propidium iodide staining, a compound that only stains necrotic cells. As Figure 37 shows, there was no significant difference between the viable cells in each group of monocytes, demonstrating that the anaphylatoxins do not induce necrosis or increased cell death in the conditions assessed here.

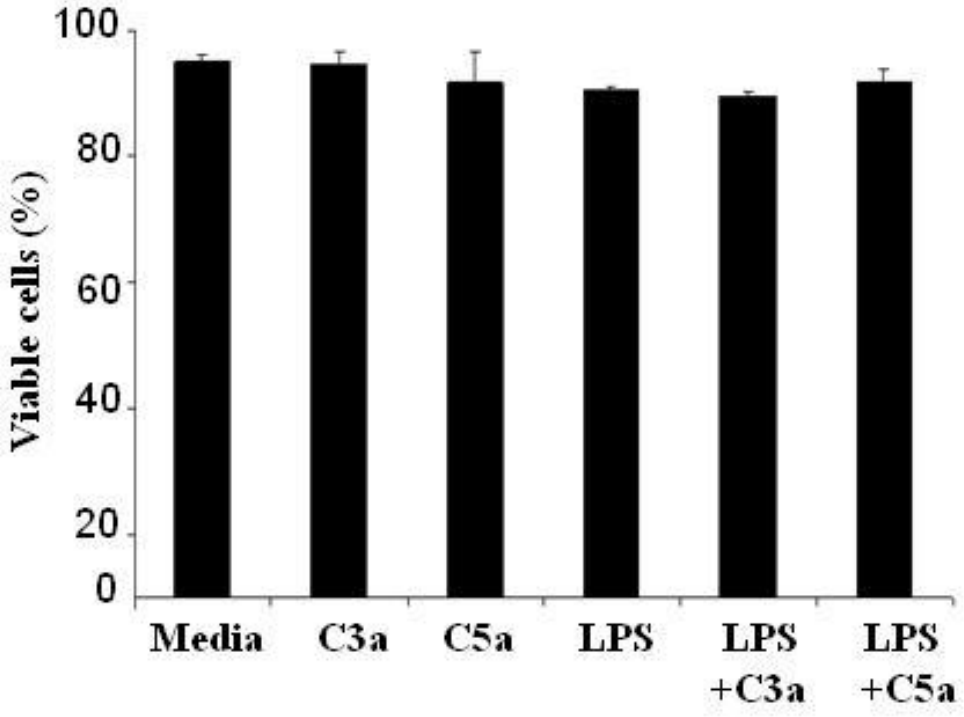


Figure 37. LPS and/or anaphylatoxin treatment of monocytes does not affect cell viability. Freshly purified monocytes were activated as depicted (LPS, 100 ng/ml; C3aR agonist, 25 μM; recombinant human C5a, 50 nM) and cell viability measured by propidium iodide staining after overnight stimulation. Data shown represent the mean ± SD (n=3).

Thus, the lack of observed changes in viability in monocytes following their stimulation with LPS and anaphylatoxins makes it unlikely that the ATP release from these cells is a consequence of their death.

8.3.8 Release of ATP is increased in cells stimulated with C3a and LPS

To confirm that increased Panx1 expression indeed leads to higher amounts of extracellular ATP following C3a stimulation, the ATP levels were measured in monocyte cell culture supernatants after overnight stimulation with 25 μM C3a agonist, 100 ng/ml LPS, or both using luciferase assay described in section 8.2.7. As expected, LPS-activated and LPS+C3aR agonist-activated cultures contained increased extracellular levels of ATP. Interestingly, ATP levels were also increased in monocyte cultures that had been activated with the C3aR agonist alone. This indicates that C3aR engagement indeed modulates ATP availability but a second TLR-mediated signal is required for the increase in IL-1β production (Figure 38).

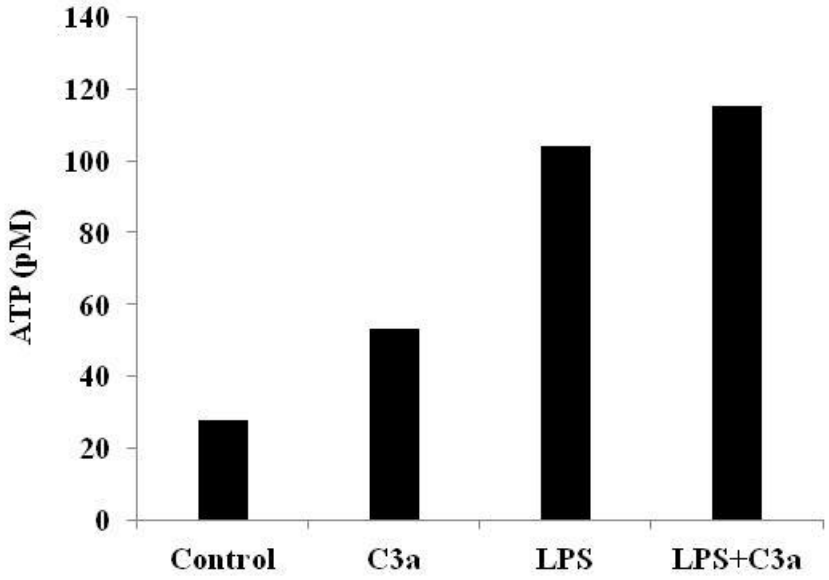


Figure 38. ATP levels are increased following C3a and LPS stimulation. Freshly isolated monocytes were stimulated overnight with C3a agonist (25 μM), LPS (100 ng/ml) and their combination. ATP was measured in the cell supernatant. This is the result of one representative experiment from 3 separate experiments on different donors (statistics was not performed due to high donor variation).

The data shown so far demonstrate that C3a but not C5a can increase LPS-mediated IL-1β production *in vitro* in human blood-derived monocytes. Mechanistically, C3aR-mediated signals increase the expression or conformation of Panx1 on the cell surface. This leads to the efflux of ATP into the extracellular space and increases signaling potentially via the P2X7 receptor, which translate into amplified inflammasome activation and IL-1β secretion.

8.3.9 C3a-mediated increase in monocytic IL-1β production is caspase-1 dependent

Although some studies have shown the existence of a caspase-1-indepenent pathway of IL-1β production (Mayer-Barber et al., 2010, Provoost et al., 2011), as discussed earlier, production of active IL-1β has been shown to be mostly dependent on caspase-1 activation. I was interested to find out if anaphylatoxin induced IL-1β production is dependent on inflammasome and caspase-1 activation.

To investigate if increased IL-1β production induced by C3aR signaling is also mediated by a caspase-1-dependent action, two types of experiments were performed: one to measure the caspase-1 activity in monocytes that were exposed to C3aR activation alone or in conjunction with LPS, and the other, to assess the IL-1β production capacity following caspase-1 blockade in cells treated with and without C3aR agonist.

The first experiment was performed using monocytes stimulated with C3aR agonist, LPS or both. Caspase-1 activity was measured at 2 hours, 4 hours and after overnight stimulation. As shown in Figure 39, while C3aR engagement alone led to only a marginal increase in caspase-1 activation, there was a significant increase in caspase-1 activity after 4 hours of LPS and LPS+C3aR agonist stimulation (the increase in

caspase-1 activity was most prominent at 4 hours. At 2 hours there was an increase in caspase-1 activity but not as significant as the 4 hour reading and the increase was not detectable in the overnight stimulated samples suggesting that the activity occurs early after LPS+/-C3aR agonist stimulation. Addition of both LPS and C3a further increased the activity of caspase-1 in monocytes. These data were the first indication that C3aR-mediated signals channel indeed into the caspase-1 activation pathway (Figure 39).

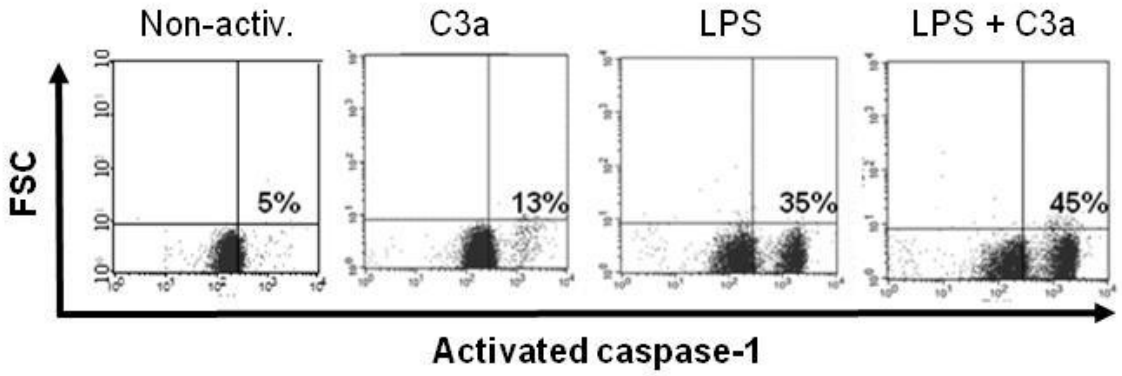


Figure 39. C3aR stimulation increases LPS-mediated caspase-1 activation. Monocytes were activated as indicated for 4 hours and active caspase-1 measured by intracellular FACS analysis using the Green FLICA caspase-1 assay kit. Shown is one representative of three similarly performed experiments.

In a complementary set of experiments, the effect of a specific caspase-1 inhibitor on LPS and C3a-induced IL-1β production was assessed. The caspase-1 inhibitor was added to cultured monocytes 15 minutes prior to their stimulation with C3a and/or LPS. Cells were incubated at 37°C incubator overnight and IL-1β production was measured in supernatants collected the next day. Although caspase-1 inhibition showed a trend in reducing the LPS-mediated production of IL-1β from monocytes, the data did not reach significance. The non-significant effect of caspase-1 inhibition in LPS-stimulated monocytes is likely due to the large variability between the donors creating the wide standard deviation (as I have observed good inhibition in some donors). In contrast, caspase-1 inhibition had a consistently significant inhibitory effect on C3a mediated IL-

1β production (Figure 40). In summary, the data from the last two figures support the notion that TLR4 and C3aR activation-mediated signals on monocytes synergize and result in an increase in caspase-1 activation leading to amplified production of IL-1β.

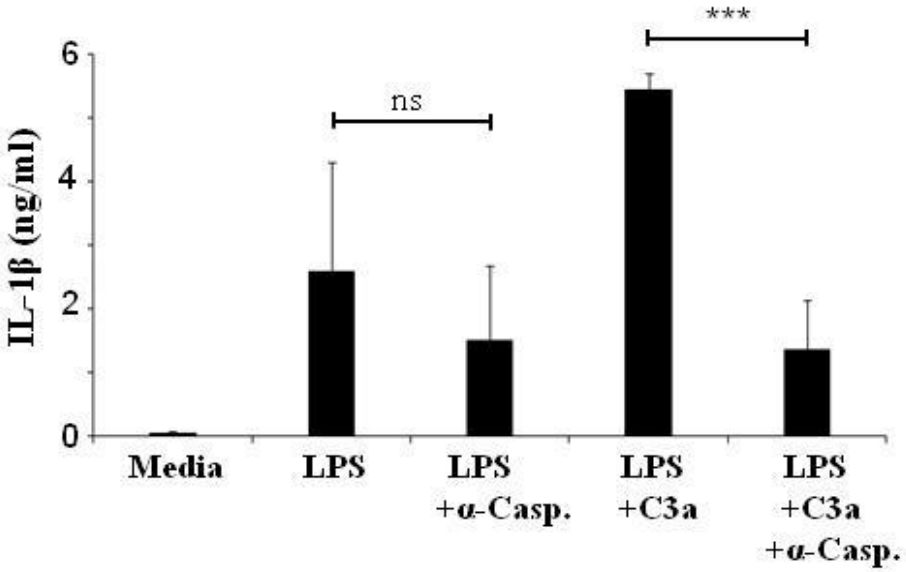


Figure 40. Inhibition of caspase-1 reduces C3a-mediated increase in monocytic IL-1β production. Inhibition of caspase-1 activity signific antly decreases LPS/C3a-mediated IL-1β production. Freshly purified monocytes were left untreated or treated with a caspase-1 inhibitor (20 μM) 15 min prior to addition of LPS (100 ng/ml) and/or C3aR agonist (25 μM). IL-1β was measured by ELISA after overnight incubation. Data shown represent mean ± SD (n=3). *, $p < 0.05$; **, $p < 0$ .005; ***, $p < 0.001$; ns, statistically not significant.

8.4 Discussion

The interplay between innate and adaptive immunity has been the subject of heightened discussion in various disease models. The past few years has seen a significant increase in research focusing on this connection, with the hope of finding ways to modulate the immune response. Studies on complement have been a major element in this path, as the

role of complement components have been extended from participating in inflammatory pathways to having a significant influence on the adaptive immune response.

Monocytes and macrophages - well known elements of the innate immune system - are known to affect the development of adaptive immunity. A growing number of experiments have demonstrated that the cells on the front line of the immune system often require more than one signal to escalate the immune response which prevents unnecessary activation and tissue destruction. The aim of this component of my work was to test the hypothesis that anaphylatoxins act as a stimulus for monocytic IL-1β production in addition to known stimuli such as LPS and ATP. Monocytes had previously been shown to produce IL-1β with LPS stimulation and without requirement for a second stimulatory signal. Macrophages however were described to need a second stimulus in addition to LPS to produce IL-1β (Netea et al., 2009). This study not only confirms the fact that monocytes produce IL-1β with LPS stimulation, but also shows that this effect can be significantly increased with the addition of anaphylatoxin C3a. This is a highly relevant finding, as IL-1β is known to have involvement in a wide range of immune reactions, and my results show that C3a could regulate its production. Local production of anaphylatoxins has an important role in triggering a diverse inflammatory response depending on the anaphylatoxin produced and the type of immune cell present at the location. Considering their effect on a wide range of immune cells, anaphylatoxins are responsible for a broad range of immune responses such as induction of co-stimulatory molecule expression on APCs thereby affecting T cell response, regulation of Th17 induction in allergic asthma and stimulating γδ-T cells to produce pro-inflammatory cytokine IL-17 (Lajoie et al., 2010, Strainic et al., 2008, Han et al., 2011, Peng et al., 2009).

One of the key roles of IL-1β which was explored in this project was the effect on adaptive immunity, specifically Th17 induction. In view of the inflammatory role of Th17 cells in various medical conditions such as transplant rejection where complement is also known to play a role, finding a relationship between the two which can potentially tie their effects together, could have important therapeutic implications; for example modulation of complement activation could lead to reduced Th17 cell induction and tissue damage.

A recent study by Cheng and colleagues has shown that infection with *Candida albicans* is able to induce IL-1β and IL-6 production from peripheral blood mononuclear cells (PBMCs) in a C5a-dependent manner (Cheng et al., 2012) (of note, the authors did not identify the exact cellular source of these cytokines in their studies). Although in my experiment, there was no effect of C5a on the IL-1β production capacity of monocytes, I did see an increase in IL-6 production by these cells following stimulation by C5a. It is possible that PBMCs other than monocytes respond to C5a by producing IL-1β. This would be an interesting area for further study where different PBMCs are investigated separately for their cytokine production response to anaphylatoxins.

Studies in various animal models have also shown a relationship between activation of anaphylatoxin receptors and IL-1β production. In 2 separate studies, Bao and colleagues have shown in a mouse model of lupus nephritis that inhibition of C3a and C5a lead to reduced expression of IL-1β in the kidney tissue as well as reduced kidney damage (Bao et al., 2005a, Bao et al., 2005b). Relationship between C3a and IL-17 production has also been shown in other disease models such as IgE-mediated asthma where inhibition of C3aR led to reduced production of IL-17 and neutrophil infiltration in the lungs (Mizutani et al., 2012).

Although experiments in the mouse deficient in C3aR or C5aR receptor would have been ideal to investigate the exact role of these receptors in IL-1β production and induction of Th17 cells in IRI or transplant rejection, the difficulty in isolating mouse monocytes as well as the fact that Th17 cell induction in human and mice are not similar (making the results from mouse experiments not directly translatable to human condition), encouraged me to use human cells and tissue in my study.

Classification of monocytes into different subtypes has added a level of complexity to the investigations of their role in immune response. There are inconsistent reports on the cytokine production ability of different monocyte subtypes in response to inflammation. A recent study has shown that non-classical monocytes which have high CD16 and relatively less CD14 expression ($CD14^{+}CD16^{++}$) are the main source of cytokine production (such as IL-1β and TNF-α) upon stimulation with LPS (Wong et al., 2011). However in another experiment, these monocytes were shown to produce low level of cytokines in response to LPS but produce higher amounts of IL-1β and TNF-α following activation of TLR7 and TLR8 by viruses and nucleic acids (Cros et al., 2010). In this study, I did not sort the monocytes into their various subtypes. Given the data observed and the fact that I had sorted the monocytes with their CD14 expression, one would expect that the classic monocytes ($CD14^{++}CD16^{+}$) are the principal responders to C3aR engagement. This needs to be formally addressed and would make an interesting subject for future studies.

I have identified in this study the role of C3aR engagement in inducing IL-1β production from monocytes and the consequent induction of Th17 cells by the cytokine milieu resulting from TLR4 and C3aR activation of these cells. This could explain the pathophysiology of certain autoimmune and inflammatory diseases such as asthma and rheumatoid arthritis in which anaphylatoxins, monocytes and Th17 cells have been

shown to play a role. Demonstration of the inflammatory component C3a in the kidney tubules as well as IL-17 and $CD4^+$ T cells in the peritubular area of rejecting kidney transplants suggests that local production of C3a in the injured organ could act as a stimulus for production of IL-1β from infiltrating monocytes which then help differentiate the $CD4^+$ T cells to IL-17 producing cells. This is in addition to the presence of damage-associated ligands for TLR4, such as high-mobility group box 1 protein, which has been reported to be present in the donor organ (Kruger et al., 2009). Understanding the interaction between the anaphylatoxin, monocyte and Th17 cells in this context can help us identify better targeted therapies for transplant rejection. Increased expression of pannexin-1 channel following C3a and LPS stimulation as well as increase in extracellular ATP in this condition indicates that activation of C3aR in conjunction with LPS leads to opening of the channel and release of ATP, which in turn activates the purine channel P2X7 and activates caspase-1, the key enzyme responsible for cleavage of pro- IL-1β. I would like to stress that although the *in vivo* significance of my findings clearly need to be better addressed, this is the first description of a connection between anaphylatoxin generation and channel regulation. One could imagine that such novel crosstalk may extend also to other channel systems, such as ion channels for example – a potential interesting area for future investigations.

Understanding the mechanism of this inflammatory pathway is critical for designing treatments which can modulate the pathway at different levels depending on the desired target and outcome. It should be noted that the diverse role of anaphylatoxins and IL-17 makes their use as therapeutic targets quite challenging. Although anaphylatoxins and IL-17 have been recognized as pro-inflammatory factors and many studies have shown beneficial effects of their inhibition in a number of inflammatory diseases, evidence is emerging that in certain circumstances these pro-inflammatory factors might have anti-

inflammatory effects and even contribute to tissue healing and repair. A recent study has shown that C5a can induce production of IL-10 and inhibit production of IL-17 in a mouse model of endotoxic shock resulting in limitation of inflammation (Bosmann et al., 2012). In the nervous system, despite C5a being suggested as a contributor to neurodegeneration in Alzheimer's disease (Farkas et al., 2003), several studies have indicated its neuroprotective capacity, such as preventing glutamate-mediated apoptosis in the neural tissue (Mukherjee et al., 2008, Osaka et al., 1999). In mixed cultures of neurons and astrocytes, C3a has been shown to protect neurons from NMDA (*N*-methyl-D-aspartate) induced cytotoxicity in a dose dependent manner (van Beek et al., 2001). C3a and C5a have also been shown to be required for liver tissue regeneration following toxic injury (Markiewski et al., 2004) and partial hepatectomy via IL-6 signaling (Strey et al., 2003, Markiewski et al., 2009).

Similarly, despite well known pro-inflammatory capacity of IL-17 in diseases such as rheumatoid arthritis and inflammatory colitis, some recent studies have now indicated a possible anti-inflammatory role for this cytokine. In a rat model of auto-immune uveitis injection of recombinant IL-17, reduced the disease activity (Ke et al., 2009) although the same disease model in mice was shown to be dependent on the presence of IL-17^+ T cells (Peng et al., 2007). In parasitic infections such as infection with *Trypanosoma cruzi* (*T. Cruzi*), IL-17-/- mice were shown to have longer parasitaemia and worse outcome compared with WT control suggesting that IL-17 is required for elimination of parasites (Miyazaki et al., 2010) while, activation of IL-17 receptor in the same disease model was shown to attract to the site of infection neutrophils that were not only responsible for elimination of the parasite but also produced IL-10, which controls the inflammatory response and prevents tissue damage (Tosello Boari et al., 2012). Furthermore, in patients who develop cardiac inflammation following *T. cruzi* infection,

higher levels of IL-17 was shown to correlate with better cardiac function, implying its protective role from myocardial injury (Magalhaes et al., 2013).

Several clinical trials are now studying the effect of anti-IL-17 treatment in autoimmune diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel disease with some positive results although longer follow up duration is required to check for long term safety of this treatment (Miossec and Kolls, 2012). Inhibition of IL-1 by using recombinant IL-1Ra known as Anakinra has been investigated in clinical trials for treatment of rheumatoid arthritis and has demonstrated beneficial effect in some group of patients (Furst, 2004). Of relevance to my work, IL-1β targeted therapy has been recently patented for use in organ transplantation to improve harvested organ viability and allograft tolerance (Wanderer, 2013).

Further investigations into the role of anaphylatoxins and IL-17 in tissue damage and regeneration following IRI are required before therapeutic targets are set.

Figure 41 summarizes the theory behind this part of my thesis.

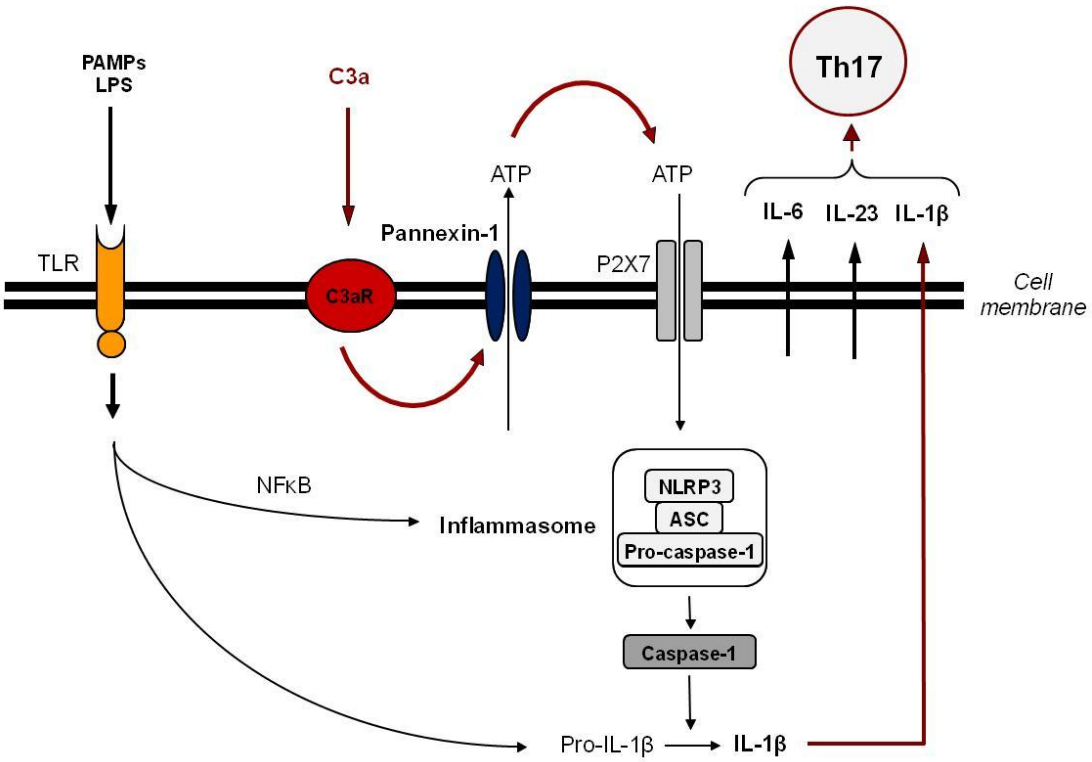


Figure 41. Proposed mechanism of C3a-dependent IL-1β production in monocytes. Co-stimulation of C3aR and TLR4 receptor in monocytes leads to activation of the pannexin-1 channel and release of ATP out of the cell which then activates (potentially) P2X7 receptor resulting in activation of inflammasome and caspase-1. Caspase-1 cleaves pro- IL-1β to its active form. Release of IL-1β in addition to IL-6 and IL-23 which are secreted in response to TLR4 stimulation, provide a milieu for the $CD4^+$ T cells to differentiate to Th17 cells.

8.5 Conclusion

I started the path of my thesis looking for the role of complement in kidney IRI. Seeing many patients with kidney failure who suffer on dialysis or encounter deterioration in their kidney transplant function, my aim was to look for a way that the life of the transplanted organ could be extended. It was encouraging to find that MASP-2 could have a potential role in kidney IRI. Although in my experiment, use of antibody did not

show a protective effect, the experiment was small and subject to variations that might be possible to remedy in further work.

My findings of the effect of C3a on monocytes and the potential to influence T cell differentiation through a defined mechanism could influence our approach to investigation and treatment of kidney transplant rejection. As described before, differences in the expression of complement components could be an indicator for a patient's risk of developing organ rejection (Naesens et al., 2009). Identifying these patients and tailoring their anti-inflammatory or immunosuppressive treatment could improve their transplant outcome.

Defining therapeutic targets has often lagged years behind any scientific findings, due to ethical considerations. However, based on the results of this project, use of therapies which are already in use in humans such as IL-1β inhibitor could be an option in preventing transplant tissue damage.

9 Future work

There are a number of interesting topics that can be followed up from this project:

- To identify the triggering factor for LP activation following IRI
- To identify through which route the LP activates C3 independent of C4
- To asses if MASP-2 inhibitor peptide prevents kidney IRI
- To investigate which monocyte subgroup responds most to anaphylatoxins and if this has an effect on the induction of T cells
- To study the effect of complement in tissue regeneration/repair following IRI
- To assess the effect of IL-1β inhibition in transplant rejection

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