CLONING, EXPRESSION AND CHARACTERISATION OF THE GLOBULAR HEAD REGIONS OF MOUSE C1Q

A thesis submitted for the degree of MPhil at Brunel University

by

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Declarations

I, Abhishek Shastri, hereby certify that this thesis has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Signature of candidate:

Signature of supervisor:

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Abstract

The classical pathway of complement system, activated by immune complexes, involves binding of globular heads of C1q to the Fc regions of aggregated IgG or IgM. At the C-terminal region of C1q, is globular head region (gC1q) where each globular head is composed of one A (ghA), one B (ghB) and one C (ghC) chain. In order to understand modularity of gC1q region of mouse C1q and to generate useful reagents for *in vivo* studies, we have expressed these recombinant individual heads of mouse C1q in *Escherichia coli* as soluble proteins linked to maltose-binding protein (MBP). This included RNA extraction and cDNA synthesis from mouse spleen, using appropriate primers, full-length mouse C1q A, B and C chains were amplified first. Following this, using the appropriate full length C1q chains, globular heads of mouse C1q A, B and C chains were amplified (mghA, mghB and mghC respectively). We have examined their interaction with heat-aggregated mouse IgG. These fusion proteins (MBP-ghA, -ghB, and -ghC) were found to bind differentially to mouse IgG. ghA and ghB were found to bind highest with IgG. IgG subtypes were purified and their interaction with native human C1q was also studied. Dose-dependent binding was observed. Interaction of recombinant globular heads of C1q with mouse IgM was also examined. ghC was found to bind highest with IgM although ghB and ghA also showed binding to IgM. The ability of these recombinant heads to inhibit complement-mediated lysis was also examined. Both MBP-ghA and MBP-ghB also inhibited C1q-dependent hemolysis of IgG sensitized sheep erythrocytes. Interaction and binding of these globular heads with recombinant prion peptide was also studied. The individual globular heads of mouse C1q were also found to bind to recombinant mouse PrP peptide which will aid in better understanding of pathogenesis of prion disease. Further studies involved interaction of recombinant mouse globular heads of C1q (mghA, mghB and mghC) with pentraxin PTX-3. mghB and mghC showed highest binding (mghB>mghC) to PTX3 as compared to mghA. The study of recombinant gC1q region heads will help in understanding the effects of PTX3 activation on the complement system. Collaborative studies examined the most variable residues in mouse gC1q region as well as alignment of mouse gC1q region with human gC1q region. The expression and functional characterisation of individual mouse gC1q domains has allowed to examine specificity and selectivity of individual globular heads of mouse C1q to known ligands. Production of mouse gC1q domains will be helpful in antibody mapping of anti-C1q autoantibodies recognising gC1q region.

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List of abbreviations

MBP: Maltose-binding protein

mghA: Recombinant globular head region of A chain of mouse C1q fused to MBP

mghB: Recombinant globular head region of B chain of mouse C1q fused to MBP

mghC: Recombinant globular head region of C chain of mouse C1q fused to MBP

PRR: pattern-recognition receptors

PAMP: pathogen-associated molecular pattern

DAMP: damage-associated molecular pattern

MAC: membrane attack complex

MBL: mannan-binding lectin

MASP: MBL-associated serine protease

fH: factor H

C4BP: C4-binding protein

PTX3: Pentraxin 3

CRP: C-reactive protein

SAP: Serum amyloid-P

HIV-1: Human immunodeficiency virus-1

HTLV-1: Human T-cell lymphotropic virus

CNS: central nervous system

SLE: systemic lupus erythematosis

AD: Alzheimer's disease

PD: Parkinson's disease

PrP: Prion protein

gC1q: globular head domain of C1q

IgG: Immunoglobulin G

IgM: Immunoglobulin M

SRBC: sheep red blood cells

EA cells: SRBC sensitised with anti-sheep IgG antibody

EDTA: Ethylenediaminetetraacetic acid

DGVB: dextrose gelatin veronal buffer

DPBS: Dulbecco's phosphate buffer saline

PCR: polymerase chain reaction

SDS-PAGE: sodium dodecyl sulphate- polyacrylamide gel electrophoresis

LB: Luria-Bertani media

IPTG: Isopropyl β-D-1-thiogalactopyranoside

1. Introduction

1.1 Innate immunity and the complement system

Innate immunity is the first line of defence against the invading pathogens and usually offers resistance within the first few hours to days (0-96 hours) of an infection (Murphy, 2012). It however does not possess the ability of specific immunity via recognition of pathogens viz. maintained by the adaptive immune system. Some of the components of first line of defence include epithelium (skin, gut, lungs) that acts as a physical barrier and also produce several kinds of antimicrobial enzymes and peptides namely lysozyme, defensins, mucin, lectin (Murphy, 2012). Once these are breached, next the pathogens come into contact with the complement system. Other components of innate immunity include the pattern-recognition receptors (PRR). These recognise not only exogenous pathogen-associated molecular pattern (PAMP) but also endogenous modified molecules called damage-associated molecular pattern (DAMP). The innate immune system launches inflammatory and regulatory responses with help of receptors, cells like phagocytes (macrophages) and mast cells, through different pathways, complement system and production of cytokines and chemokines to counteract infection, injury and maintenance of tissue homeostasis.

The complement system

The complement system comprises of more than 30 proteins in the serum as well as membrane-bound receptors and regulators. The complement system consists of 3 different initiating or activation pathways culminating into a final common lytic pathway, leading to the formation of membrane attack complex (MAC). MAC are pores that penetrate cell membrane (lipid bilayers) of pathogens or abnormal cells, thereby causing their lysis.

The three initiating pathways are called (i) classical pathway which is mostly antibody mediated (C1q being the first subcomponent) and is activated by C1 complex (C1q-C1r-C1s); (ii) alternative pathway (AP) which is activated spontaneously involving low-level hydrolysis of C3 to C3 (H_20); and (iii) lectin pathway where activation occurs through binding of a carbohydrate pattern present on microorganisms called mannan, with mannan-binding lectin (MBL) and Ficolins (ficolin-1, -2 and -3). They circulate in the serum in combination with zymogen serine proteases called MBL-associated serine proteases (MASPs) (Kishore and Reid, 2000; Shastri et al., 2013).

All the 3 pathways ultimately converge to lead to formation of C3 convertase. C3 convertases then cleaves C3 into C3a and C3b. This C3b binds to C3 convertase and leads to the formation of C5 convertase. This C5 convertase cleaves C5 into C5a and C5b. C3a and C5a are called anaphylatoxins and are chemoattractants. The C5b formed associates with C6, C7, C8, and C9 to form MAC (Shastri et al., 2013). The functions of the complement system include opsonisation of pathogens, direct lysis of foreign cells, chemotaxis and activation of leukocytes, and clearance of apoptotic cells. The complement system is kept in

check by regulators in order to prevent overactivation leading to damage to tissues and autoimmune diseases. The regulators can be grouped into fluid-phase: factor H (fH) and properdin for alternative pathway, C1 inhibitor and C4b-binding protein (C4BP) for classical and MBL pathway; host cell membrane-bound: CR1, CR2, CD55, CD46, CD59; cell surface-attached complement regulators: fH, factor H-like protein 1 (FHL-1), C4BP and clusterin (Shastri et al., 2013). For certain ligands, factor H can also regulate C1q-mediated classical pathway (Kishore and Sim, 2012, Tan et al., 2010; Tan et al., 2011; Kang et al., 2012).
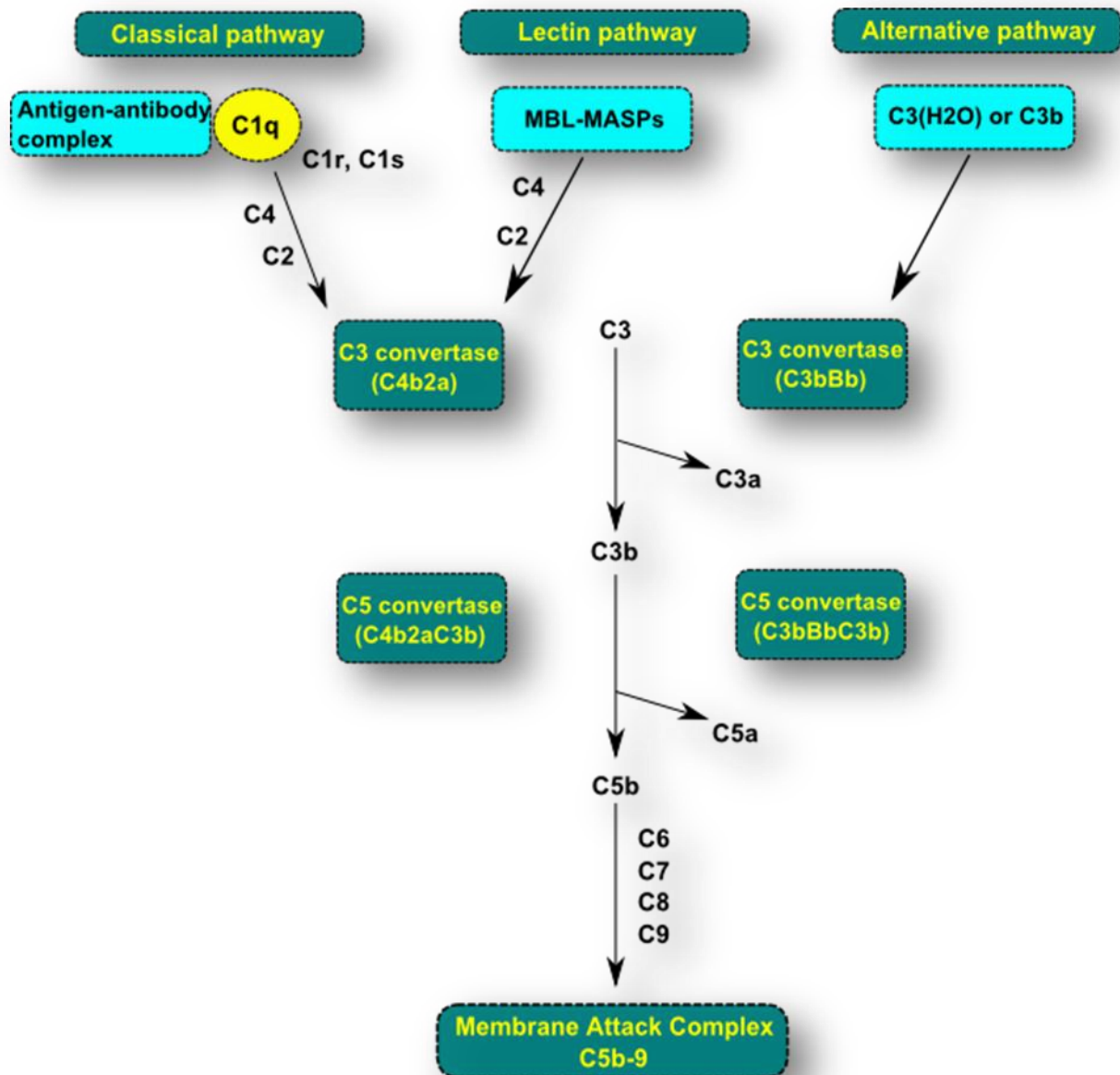
Figure 1. The complement system consists of three activating pathways namely classical, alternative and lectin pathways. The classical pathway is activated by antigen-antibody complexes binding to C1q, while the lectin pathway is activated by binding of mannose (present on microbes) to membrane associated serine proteases (MASPs). This leads to formation of a common C3 convertase (C4b2a). The alternative pathway is spontaneously activated by low-level hydrolysis of C3 to C3(H$_2$O), leading to formation of C3 convertase (C3bBb). The C3 convertases leads to formation of C3b and C3a. Then, C3b is involved in the formation of C5

converatses which leads to formation of C5b and C5a. C3a and C5a are anaphylotoxins. C5b combines with C6, C7,C8 and C9 to form membrane attack complex.

C1q

C1q is a 460 kDa molecule and is the first subcomponent of the classical complement pathway. It is composed of 18 polypeptide chains (six A, six B and six C chains). The A chain (223 residues), B chain (226 residues) and C chain (217 residues) consist of a short N-terminal region (~ 3 to 9 residues), collagen-like region (CLR; ~ 81 residues) and C-terminal globular head region (gC1q; ~ 135 residues) (Sellar et al., 1991).

There are 4 cysteine residues at positions (numbering as per B chain) 4, 135, 154 and 171. The cysteine residue at position 4 is involved in inter-chain disulphide bridge, leading to formation of A-B and C-C sub-units. The other three cysteine residues are thought to form one intra-chain disulphide bond (6 A-B and 3 C-C dimer subunits). The CLR region of A and B chains of A-B subunit along with CLR region of C chain of C-C subunit for a triple-helix ABC-CBA unit and three such units associate to form the hexameric structure of C1q (Reid and Porter, 1976; Kishore and Reid, 1999; Kishore et al., 2003). C1q is a pattern-recognition receptor (PRR) and its structure resembles other PRRs such as MBL, ficolin, surfactant protein (SP)-A, SP-D (Nayak et al., 2010).

The structure of C1q shows a β sandwich fold with jelly roll topology, similar to that described for tumour necrosis factor and gC1q family, leading to recognition of C1q/TNF superfamily (Kishore et al., 2004). The structure of gC1q also shows presence of a Ca^{2+} at the top of the assembly (Gaboriaud et al., 2012). The gC1q module is found in a number of other non-complement proteins (Kishore and Reid, 2000) such as collagens type VIII and type X, Acrp-30 (adipose-specific protein), saccular collagen (found in inner ear), elastin microfibril interface-located protein (extracellular matrix glycoprotein), precerebellin and multimerin (Yamaguchi et al., 1989; Ninomiya et al., 1986; Scherer et al., 1995; Hu et al., 1996; Davis et al., 1995; Doliana et al., 1999; Urade et al., 1991; Hayward et al., 1995).

Apart from classical complement pathway activation (ligands being IgG, IgM, β-amyloid), some of the other functions include immune-complex clearance, bacterial cells clearance, binding and inactivation of viruses, induction of pro-inflammatory cytokines, clearance of apoptotic cells and coagulation (Nayak et al., 2010). C1q is also implicated in neuroinflammation, scrapie pathogenesis, T-cell and B-cell development, pregnancy and central nervous system (CNS) development (Shastri et al., 2013).

Some of the disorders associated with aberrant C1q activity are listed in Table 1.

Table 1: List of disorders associated with aberrant C1q activity (Shastri et al, 2013)

Diseases	Role/Involvement of C1q
Systemic lupus erythematosus	Deficiency of C1q Autoantibodies against C1q and globular heads of C1q
Nephropathy	Deposition of C1q in mesangium or glomerulus of kidneys leading to nephritis or nephrotic syndrome
Alzheimer's disease	Dual role of neuroprotection and neurodegeneration
Familial dementias	Complement pathway activation
Prion diseases	Uptake and recognition of prion protein
Traumatic brain injury	Upregulation of C1q
Cerebral malaria	Increased C1q activity & formation of MAC
Fungal infections like Aspergillosis	Increased C1q production by macrophages
Multiple sclerosis	Priming of microglia
Parkinson's disease	Uptake of neuromelanin by C1q-positive microglia
Huntington's disease	Pathological deposition in neuropathological studies
Schizophrenia	Upregulation of complement activity
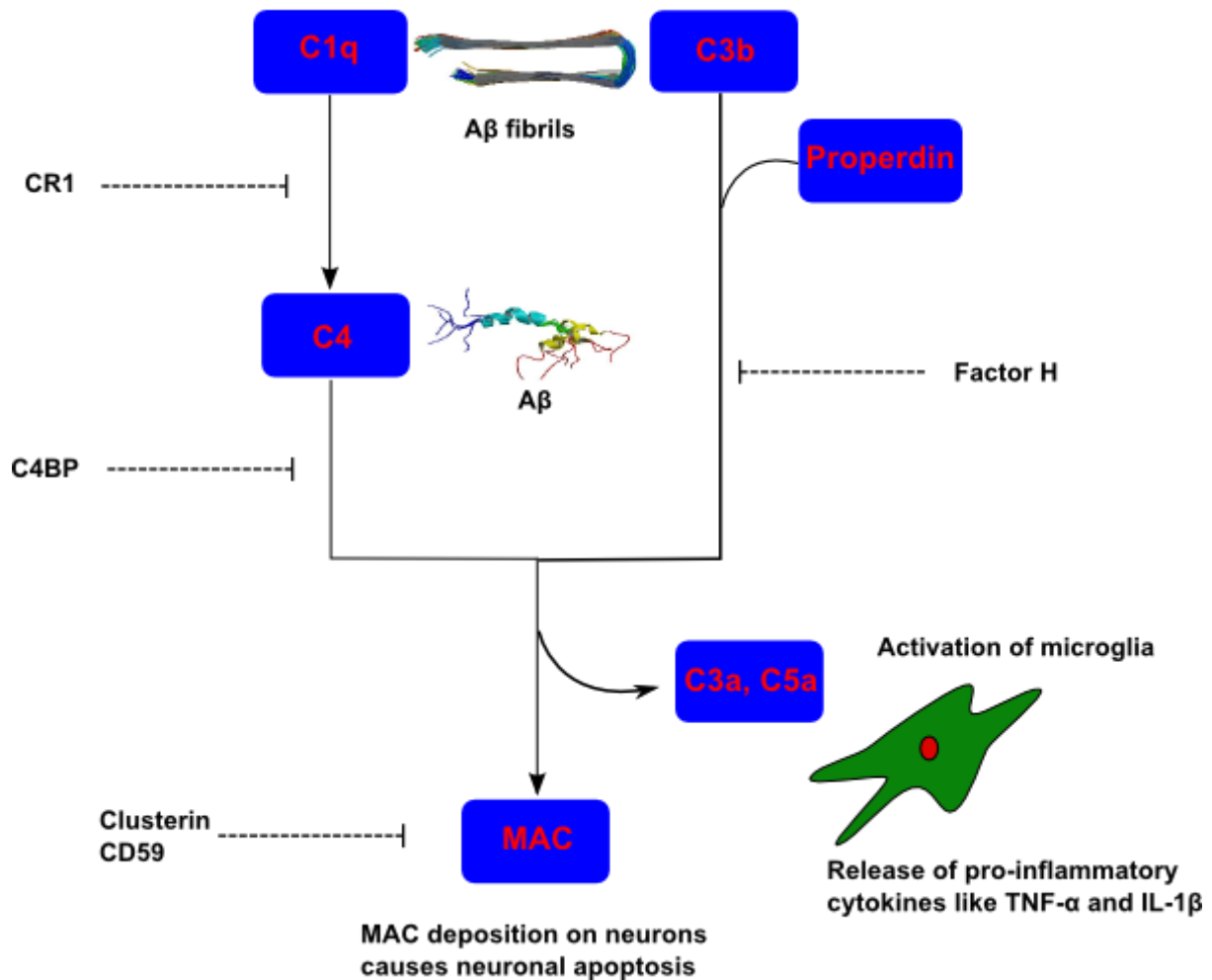
Figure 2: Association of classical and alternative pathway of the complement system with β-amyloid (Aβ). C1q and C3b bind to fibrillar Aβ[1] (see below for image reference) and activate the classical and alternative pathways respectively while C4 is activated by non-fibrillar Aβ[2] (see below for image reference). This activation of complement pathway leads to formation of anaphylotoxins C3a and C5a as well as membrane attack complex (MAC). These anaphylotoxins are involved in the activation of microglia which leads to secretion of pro-inflammatory cytokines like tumour necrosis factor-α (TNF-α) and interleukin 1-β (IL-1β). MAC deposition on neurons leads to neuronal apoptosis. Complement regulators for classical pathway involved are complement receptor 1 (CR1) and C4b-binding protein (C4BP), alternative pathway is factor H, and membrane attack complex (MAC) is stabilised by clusterin and CD59.

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[1] Image from the RCSB PDB (www.pdb.org) of PDB ID 2BEG (T. Luhrs, C. Ritter, M. Adrian, D. Riek-Loher, B. Bohrmann, H. Dobeli, D. Schubert, R.Riek (2005) 3D structure of Alzheimer's amyloid-beta (1-42) fibrils Proc.Natl.Acad.Sci.USA 102: 17342-17347).

[2] Image from the RCSB PDB (www.pdb.org) of PDB ID 1Z0Q (S. Tomaselli, V. Esposito, P. Vangone, N. A. van Nuland, A. M. Bonvin, R. Guerrini, T. Tancredi, P. A. Temussi, D. Picone (2006) The alpha-to-beta conformational transition of Alzheimer's Abeta-(1-42) peptide in aqueous media is reversible: a step by step conformational analysis suggests the location of beta conformation seeding Chembiochem 7: 257-267).

Table 2: Known ligands of C1q (adapted from Kishore and Reid, 2000)

Immunoglobulins	IgG (IgG3, IgG1, IgG2)	Globular head region B of C1q is the main IgG-binding molecule for C1q. Weak interaction is observed in the binding of C1q to Fc region (non-aggregated) but the interaction increases thousand-fold when the simulation on immune complexes (closely spaced Fc portions) occurs. The Fab portion also interacts with C1q.
	IgM	Globular head region C of C1q is most specific in binding to IgM. Monomeric form of IgM does not bind C1q. The hexameric and pentameric forms of IgM bind to C1q with the efficiency of former being 100 times more than the latter in activation of complement system.
Non-immunoglobulins	CRP	C1q binds to CRP through the globular head region with pentameric form of CRP. CRP binds to nuclear debris and by binding to C1q, helps in clearance of chromosomal material from necrotic cells.
	SAP	SAP binding to C1q helps in preventing antinuclear autoimmunity by helping in controlling degradation of chromatin.
	PTX3	PTX3 interacts with C1q via the globular head region and interacts with the tetrameric C1q-C1r2-C1s2. This helps in uptake of apoptotic cells by phagocytes and also amplifies response to fungal pathogens.
	Decorin	Decorin binds to the neck region of C1q and downregulates proinflammatory effects of C1q.
	Fibronectin	Fibronectin binds to C1q and play a role in formation of cryoglobulins and clearance of immune complexes.
	Lactoferrin	Lactoferrin binds to C1q and helps in uptake of Streptococci.
	Calreticulin	Calreticulin binding with C1q helps in apoptotic cell recognition and uptake as well as elimination of immune complexes.
	SIGN-R1	C1q binds to SIGN-R1 which in-turn binds to *Streptococcus pneumonia* and causes C3d deposition on the bacteria leading to bacterial killing.
Viruses	HTLV-1	Interaction and binding of C1q to HTLV-1 inhibits its infectivity
	HIV-1	Interaction and binding of HIV-1 by C1q results in viral neutralisation and activation of classical complement pathway
Cells	Neutrophils	Enhances phagocytosis and chemotaxis
	Monocytes	Enhances phagocytosis

	Platelets	Induces expression of adhesion molecules
	B cells	Enhances secretion of Immunoglobulins
	Fibroblasts	Enhances phagocytosis and chemotaxis
Polyanions	Heparin	Heparin binds to C1q and inhibits activation of classical pathway
	Cardiolipin	Binding of cardiolipin to C1q occurs via gC1q región, significant in ischaemic myocardium reperfusion injury
	Lipopolysa-ccharide	Gram-negative bacteria actívate complement pathway in an antibody-independent manner by binding via LPS on bacteria (gC1q región is involved in this binding and recognition)
	Cardiolipin	Binding of cardiolipin to C1q occurs via gC1q región, significant in ischaemic myocardium reperfusion injury
	Chondroitin suplate	Secreted by activated platelets, it activates complement system and is significant in myocardial ischaemia
	Phosphati-dylserine	Phosphatidylserine provides "eat-me" signal on apoptotic cells and helps in apoptotic cell clearance
	DNA	C1q binds to DNA via gC1q región and is involved in apoptotic cell clearance and play a key role in flare-ups as well as pathogenesis of SLE

Structure of globular head region of C1q and modularity
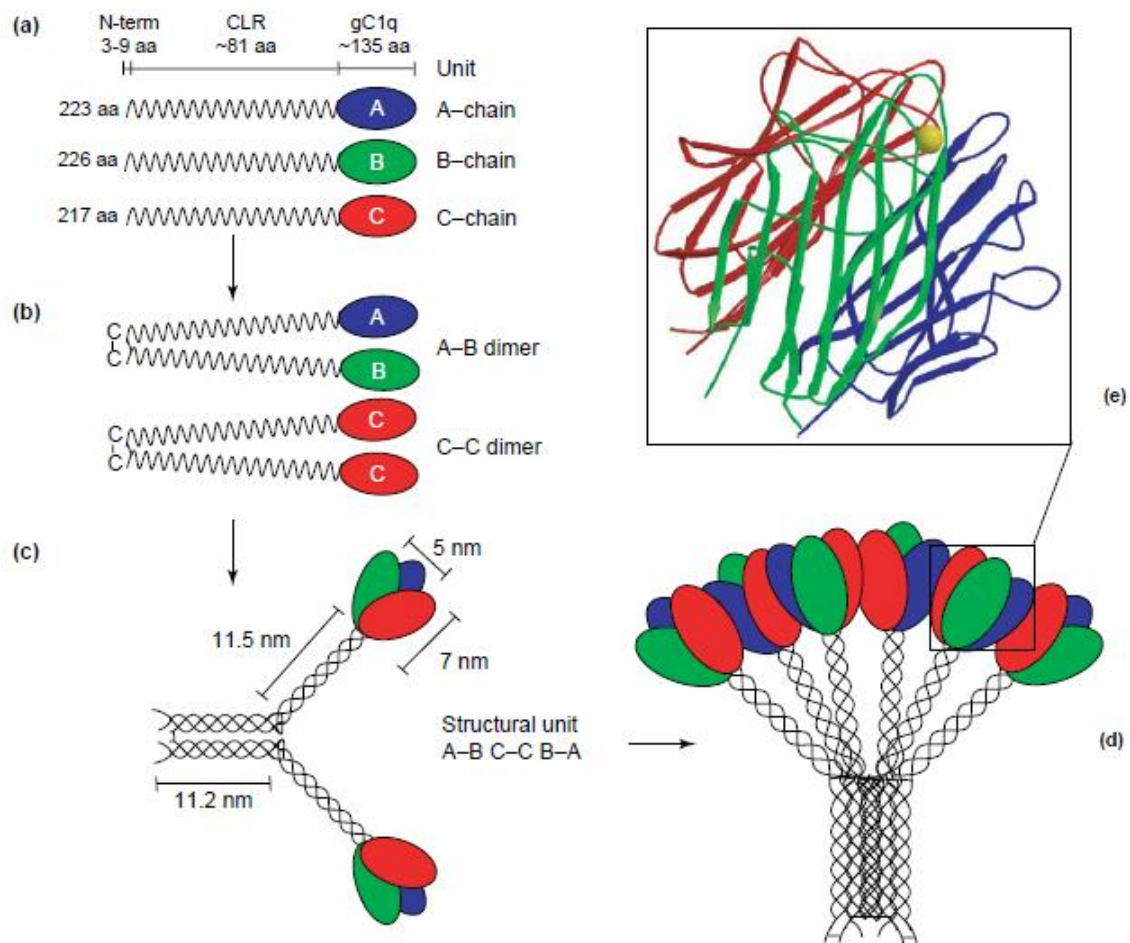
Figure 3: Structural organization of the C1q molecule. C1q (460 kDa) is composed of 18 polypeptide chains (6A, 6B and 6C). (a) The A, B and C chains each have a short N-terminal region (containing a half-cystine residue involved in interchain disulfide bond formation), followed by a collagen region (CLR) of w81 residues and a C-terminal globular region (gC1q domain) ofw135 residues. (b) The interchain disulfide bonding yields 6A–B dimer subunits and 3C-C dimer subunits. The triple-helical collagen region in the A and B chains of an A–B subunit, together with one of the C-chains present in a C–C subunit, form a structural unit (ABC–CBA), which is held together by both covalent and non-covalent bonds (c). Three of these structural units associate, via strong non-covalent bonds in the fibril-like central portion, to yield the hexameric C1q molecule that has a tulip-like structure. Image and legend Reference: Kishore U, Gaboriaud C, Waters P, Shrive A, Greenhough, T, Reid, KBM, Sim RB, Arlaud G; *Trends in Immunology;* vol. 25, No. 10, (2004).

The globular head domain of C1q (gC1q) is the C-terminal region composed of its A, B and C chains (ghA, ghB and ghC respectively). The gC1q domain has a heterotrimeric organisation and each of the three modules of gC1q can bind ligands independently (Kishore et al, 2003). ghA can bind heat-aggregated IgG and IgM as well as HIV-1 gp41-derived loop peptide. ghB binds to heat-aggregated IgG as well as β-amyloid peptide. ghC shows preferential binding to IgM as well as HTLV-1 gp21 peptide. In addition, ghB shows a predominance of positive

charges on its external face whereas ghA and ghC show a combination of basic and acidic residues on their external face (Appendix 3; Kishore et al, 2004).

Thus, the presence of modularity of the heterotrimeric assembly coupled with surface charge pattern differences and spatial orientation of the globular heads, it adds to the flexibility and versatility of the gC1q module (Kishore et al, 2004).
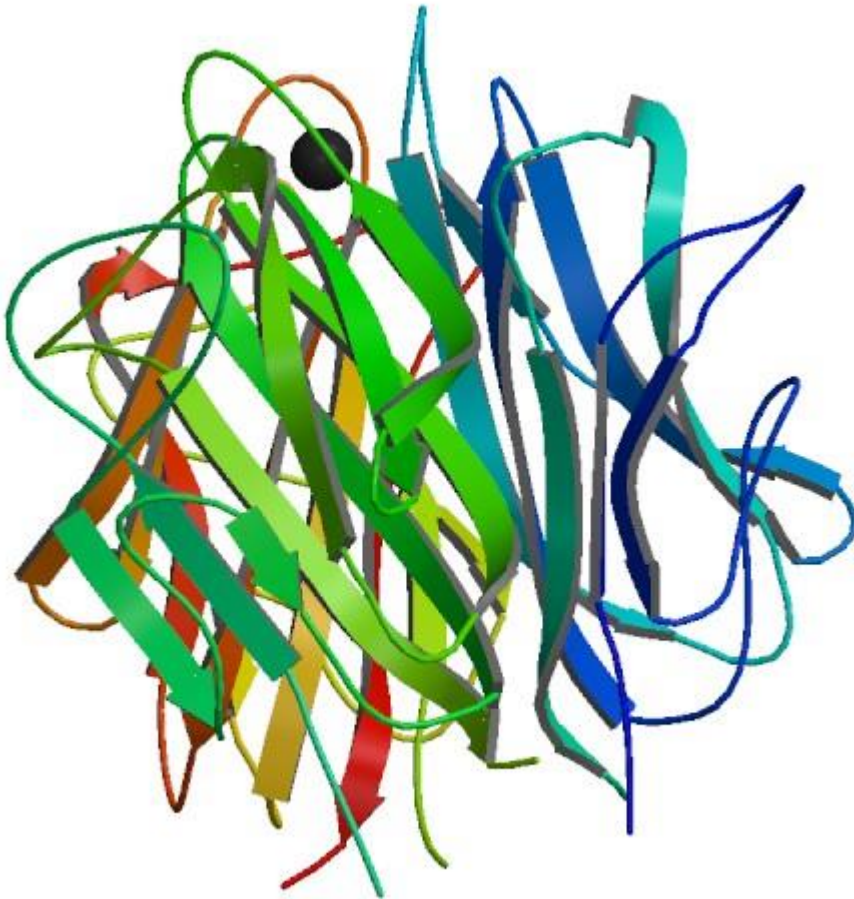
Globular Head of the Complement System Protein C1q
Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient.

Figure 4: Image from the RCSB PDB (www.rcsb.org) of PDB ID 1PK6; Gaboriaud, C., Juanhuix J., Gruez, A., Lacroix, M., Darnault, C., Pignol, D., Verger, D., Fontecilla-Camps, J.C., Arlaud, G.J. (2003); The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. J.Biol.Chem. 278: 46974-46982.

The 3-D structure of gC1q revealed a diameter of 50 Angstrom with the N-terminal and C-terminal regions of each sub-unit emerging at the base of the trimer, very close to one another. gC1q subunits were found to be arranged clockwise in the order A, B, C when viewed from the superior angle (top view) (Gaboriaud et al., 2011). The B subunit (ghB) was found to lie on the outer part of the molecule and ghA and ghC were positioned on the

inside. A free cysteine residue is conserved in gC1q domain and in addition, each subunit of C1q contains two other cysteine residues in disulphide bonds.

Binding of C1q to IgG1 and PTX3 is considered to be highly electrostatic in nature as *in vitro* studies have shown that the binding is highly sensitive to the ionic strength of the binding buffer (presence of 1.2 M NaCl abolishes interaction to a large extent) (Roumenina et al, 2006). The exposed Ca^{2+} near the apex of gC1q influences interaction of C1q with IgG1, CRP and PTX3. Mutational analysis studies have shown that Arg^{B114}, Arg^{B129} and His^{B117} are central to interaction of gC1q with IgG1. Tyr^{B175} and Lys^{C170} are central to C1q-PTX3 interaction (Roumenina et al, 2006).

IgG and IGM binding sites on gC1q are distinct but may overlap and in vitro studies have also confirmed the importance of Arg^{B108} and Arg^{B109} in initial recognition and final binding of gC1q wth IgG and IgM. Lys^{A200} and Lys^{C170} were also found to be central to C1q binding with IgG and IgM (Gadjeva et al, 2008).

Prion diseases or transmissible spongiform encephalopathies are a group of neurodegenerative disorders that are infective and are known to occur in humans, cattle and other livestock such as sheep and deer. Creutzfeldt-Jakob disease (CJD), fatal familial insomnia and kuru are known prion diseases in humans with the variant form of CJD being transmissible from meat infected with prion disease. The fibrillar form of normally present prion proteins, referred to as PrP^{Sc} (scrapie-associated prion protein), accumulates in the neurodegenerative plaques – these are cytotoxic are form protease-resistant fibrils and oligomers. Infection is propagated via oral ingestion, following which the PrP^{Sc} penetrates the intestinal epithelium, facilitated by proliferation involving follicular dendritic cells, enters the lymphatic system and finally makes its way into the CNS. C1q interacts with PrP and has been shown to involve gC1q region and help in uptake of PrP as well as activation of classical complement pathway. (Mitchell, 2013; Mitchell et al., 2007)

Pentraxins are a family of evolutionary conserved proteins pattern recognition proteins containing the carboxy-terminal 200 amino acid pentraxin domain. Based on the length of their structure, these are classified into short and long pentraxins. The short pentraxins are CRP and SAP which are acute-phase proteins. PTX3 is a long pentraxin and produced most abundantly by dendritic cells. PTX3 interacts and recognises selected pathogens such as gram-negative bacteria and *Aspergillus fumigatus*, growth factors, oophorous matrix, apoptotic cells and extracellular matrix components. PTX3 interacts with C1q via gC1q region and has also been shown to interact with recombinant globular heads of human C1q along with Tyr^{B175} and Lys^{C170} being central to this interaction (Nauta et al, 2003; Roumenina et al, 2006; Kishore and Reid, 2007).

A lot of work has been done on mouse C1q in the field of nephritis (Trouw et al., 2004a; Trouw et al., 2004b; Trouw et al., 2003), systemic lupus erythematosis (Hogarth et al., 1996; Trinder et al., 1995; Cortes-Hernandez et al., 2004), Alzheimer's disease (Fonesca et al.,

2011) and prion diseases (Klein, et al., 2001; Mabbott et al., 2001; Mitchell et al., 2007). In order to understand modularity of gC1q region of mouse C1q and to generate useful reagents for mouse work, we have expressed these recombinant individual heads of mouse C1q. We have examined their interaction with heat-aggregated mouse IgG and IgG isotypes, mouse IgM, mouse pentraxin-3 (PTX-3) and prion peptide.

Materials and methods

RNA extraction and cDNA synthesis

Total RNA was prepared from mouse spleen using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. 10 µg of total RNA was digested RNase-free DNase I (Promega), extracted once with phenol/chloroform/isoamyl alcohol (25/24/1). RNA was precipitated from the aqueous phase, washed with ethanol and finally re-dissolved in water.

For cDNA synthesis, one µg of RNA was reverse transcribed into cDNA according to the instruction manual of superscript tm first strand cDNA synthesis system for RT-PCR (Invitrogen). Briefly, 1 µl of Oligo(dT)23 anchored primers (Sigma) were added to 1µg of RNA in a final volume of 11.5 µl. The reaction mixture was incubated for 10 minutes at 70°C. After that, the temperature was decreased to 45°C and 1 µl superscript II, 2 µl RT buffer (10x), 2 µl MgCl2, 2 µl of 0.1M DTT, 1 µl dNTPs (10mM) and 0.5 µl RNasin (Promega) were added and the reaction mixer was re-incubated for another 60 minutes at 45°C, followed by 10 minutes incubation at 70°C. Finally, the temperature was dropped to 4°C. After cDNA preparation, the template RNA was digested using one µl of RNase H (Promega) and incubation at 37°C for 20 minutes.

Construction of vectors for the extracellular expression of full length recombinant mouse C1q

The plasmid used for protein expression was pSectag-B (New England Biolabs, Beverley, MA). The primers used to amplify the DNA sequences of each of the globular heads were: C1qA forward primer (FP) 5'-GGGGAATTCAGGGACCAGCCCCGGCC-3' and C1qA reverse primer (RP) 3'- GGGAAGCTTTCAGGCCGAGGGGAAAATGAGGAAT-5'; C1qB FP 5'-GGGGAATTCGGGGCTACACAGAAAGTCGCCT-3' and C1qB RP 3'-GGGAAGCTTTTACGCATCCATGTCAGGGAAAAGCA-5'; C1qC FP 5'-GGGGAATTCAAACAGAAGCACCAGTCGGTATTCACA-3' and C1qC RP 3'-GGGAAGCTTCTAGTCGGGAAACAGTAGGAAACCA-5'. Plasmid pMal-c was double digested with *HINDIII* and *EcoRI* and each PCR product was cloned as a *HindIII-EcoRI* fragment. The

polypeptide sequence thus expressed corresponded to residues of C1qA, of C1qB and of C1qC. The recombinant vectors were named as pAS-A, pAS-B and pAS-C, respectively.

Plasmid pMal-c

The vector pMAL-c is designed to produce maltose-binding protein (MBP) fusions, where the protein of interest can be cleaved from MBP with a specific protease called as Factor Xa. The MBP fusion proteins formed are expressed in the cytoplasm, and for purification purposes, the MBP is engineered to bind tightly to amylose resin column. A gene can be inserted in the restriction site of vector, in the translational reading frame of *malE* gene. The *malE* gene encodes for MBP. This provides for the fusion protein to be purified using amylose resin affinity chromatography and can then later be cleaved from MBP using the protease Factor Xa.
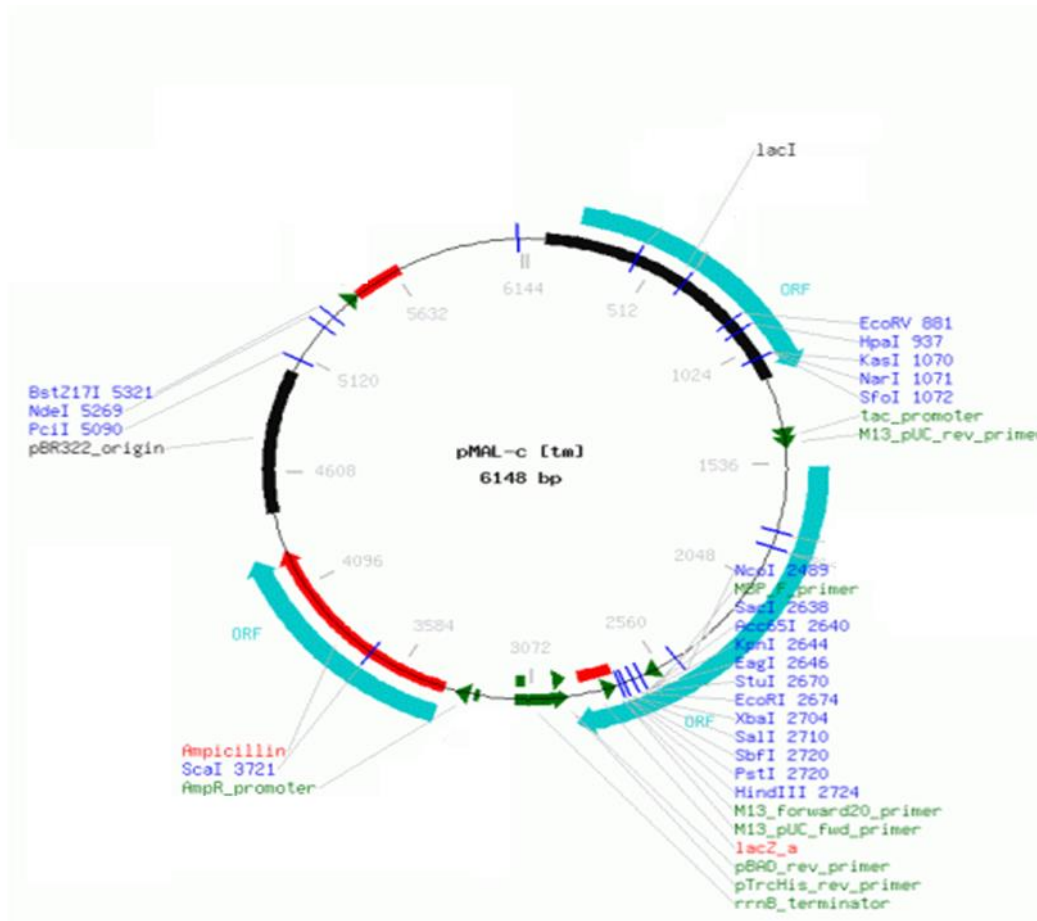

Figure 5a: Vector pMalc

The system uses the method of "tac" promoter and carries *LacZ* gene which codes for the Lac repressor. This keeps the expression from P_{tac} low in the absence of IPTG (induction). The vector consists of multiple cloning sites containing restriction sites for cloning fragments. Blunt-ended subcloning method was implemented.
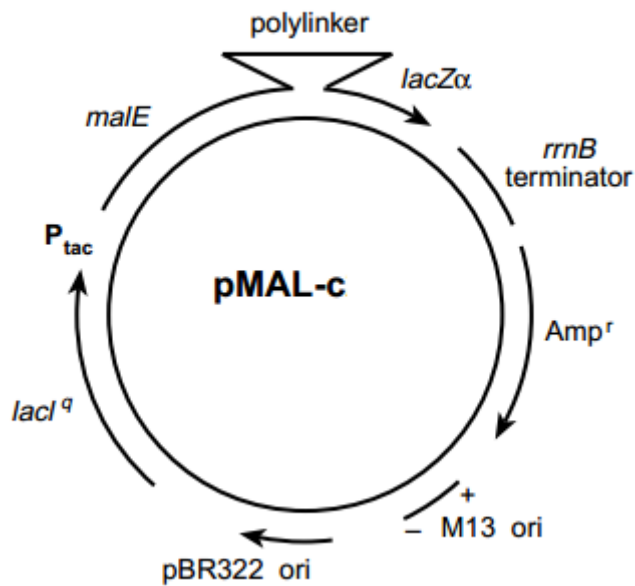
Figure 5b: Vector pMalc

<u>Full-length mouse C1q A chain</u>

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ATGGAGACCTCTCAGGGATGGCTGGTGGCCTGTGTGCTGACCATGACCCTAGTATGGACAGTGGCTGAAGATGTC
TGCCGAGCACCCAACGGGAAGGATGGGGCTCCAGGAAATCCTGGCCGCCCGGGGGAGGCCGGGTCTCAAAGGAGAG
AGAGGGGAGCCAGGAGCTGCTGGCATCCGGACTGGTATCCGAGGTTTTAAAGGAGACCCAGGGGAATCTGGCCCC
CCTGGCAAACCTGGCAATGTGGGGCTCCCAGGTCCCAGTGGTCCCCTGGGGGACAGCGGCCCCCAAGGACTGAAG
GGCGTGAAAGGCAATCCAGGCAATATCAGGGACCAGCCCCGGCCAGCTTTCTCAGCCATTCGGCAGAACCCAATG
ACGCTTGGCAACGTGGTTATCTTTGACAAGGTCCTCACCAACCAGGAGAGTCCATACCAGAACCACACGGGTCGC
TTCATCTGTGCAGTGCCCGGCTTCTATTACTTCAACTTCCAAGTGATCTCCAAGTGGGACCTTTGTCTGTTTATC
AAGTCTTCCTCCGGGGGCCAGCCCAGGGATTCCCTGAGTTTCTCTAACACCAACAACAAGGGGCTCTTTCAGGTG
TTAGCAGGGGGCACCGTGCTTCAGCTGCGACGAGGGGACGAGGTGTGGATCGAAAAGGACCCCGCAAAGGGTCGC
ATTTACCAGGGCACTGAAGCCGACAGCATCTTCAGCGGATTCCTCATTTTCCCCTCGGCCTGA
```

<u>Key</u>

 - signal peptide   - collagen-like region   -globular head region

Figure 6a. Sequence of full-length mouse C1q A chain

<u>Full-length C1q B chain</u>

```
ATGAAGACACAGTGGGGTGAGGTCTGGACACACCTGTTACTGCTGCTTCTAGGTTTTCTCCATGTGTCCT
GGGCCCAAAGCAGCTGCACCGGGCCCCCTGGCATCCCTGGCATCCCTGGGGTCCCTGGGGTTCCTGGCTC
TGATGGCCAACCAGGCACTCCAGGGATAAAGGGGGAGAAAGGGCTCCCTGGACTGGCTGGAGACCTTGGT
GAGTTTGGAGAGAAAGGGGACCCAGGGATCCCTGGGACTCCAGGCAAAGTTGGCCCTAAGGGTCCCGTCG
GCCCTAAGGGTACTCCAGGCCCCTCTGGACCCCGCGGTCCCAAAGGCGATTCTGGGGACTACGGGGCTAC
ACAGAAAGTCGCCTTCTCTGCCCTGAGGACCATCAACAGCCCCTTGCGACCGAACCAGGTCATTCGCTTC
GAAAAGGTGATCACCAACGCGAACGAGAACTATGAGCCACGCAACGGCAAGTTCACCTGCAAGGTGCCTG
GCCTCTACTACTTCACCTATCATGCCAGCTCCCGGGGCAACCTGTGTGTGAATCTCGTTCGTGGCCGCGA
TCGGGACAGCATGCAGAAAGTAGTCACCTTCTGTGACTATGCCCAGAACACCTTCCAGGTGACCACAGGT
GGGGTAGTCTTGAAGCTAGAGCAAGAGGAGGTTGTTCACCTGCAGGCCACAGACAAGAACTCCCTCCTGG
```

GCATTGAGGGTGCCAACAGCATCTTCACTGGCTTTC<u>TGCTTTTCCCTGACATGGATGCGTAA</u>

<u>Key</u>

▭ - signal peptide   ▭ - collagen-like region   ▭ -globular head region

Figure 6b. Sequence of full-length mouse C1q B chain

<u>Full length C1q C chain</u>

ATGGTCGTTGGACCCAGTTGCCAGCCTCCATGTGGACTTTGCCTGCTGCTGCTGTTTCTTCTGGCCCTAC
CACTCAGGAGCCAGGCCAGCGCT<u>GGCTGCTATGGGATC</u>CCAGGGATGCCAGGCATGCCGGGGGCCCCTGG
GAAGGACGGGCATGATGGACTCCAGGGGCCCAAGGGAGAGCCAGGAATCCCAGCCGTCCCTGGGACCCGA
GGACCCAAGGGTCAGAAGGGCGAGCCTGGCATGCCTGGCCACCGTGGGAAAAATGGCCCCAGGGGGACCT
CAGGGTTGCCAGGGGACCCAGGCCCCAGGGGGCCTCCGGGGGAGCCAGGTGTGGAGGGCCGATACAAACA
GAAGCACCAGTCGGTATTCACAGTCACCCGGCAGACCACCCAGTACCCAGAGGCCAACGCCCTCGTCAGG
TTCAACTCTGTGGTCACCAACCCTCAGGGGCATTACAACCCAAGCACAGGGAAGTTCACCTGTGAAGTGC
CGGGCCTCTACTACTTCGTCTACTACACATCGCATACGGCCAACCTGTGCGTGCACCTGAACCTCAACCT
TGCCAGGGTGGCCAGCTTCTGCGACCACATGTTCAACAGCAAGCAGGTCAGCTCCGGAGGAGTCCTCCTG
CGGCTCCAGAGGGGCGATGAGGTGTGGCTATCAGTCAATGACTACAATGGCATGGTGGGCATAGAGGGCT
CCAACAGCGTCTTCTC<u>TGGTTTCCTACTGTTTCCCGACTAG</u>

<u>Key</u>

▭ - signal peptide   ▭ - collagen-like region   ▭ -globular head region

Figure 6c. Sequence of full-length mouse C1q C chain

<u>Primers designed</u>

Key: **F**: Forward primer; **R**: Reverse primer; **T_m**: Melting temperature in degree Celsius; **bp**: Base pairs; **restriction enzyme** used is underlined

Mouse C1qA

F (<u>HINDIII</u>): GGG<u>AAGCTT</u>GAAGATGTCTGCCGAGCACCC
T_m=58   GC=62%, AT=38%   bp=21

R (<u>EcoRI</u>): GGG<u>GAATTC</u>TCAGGCCGAGGGGAAAATGAGGAAT
T_m=59   GC= 52%, AT= 48%   bp=25

Mouse C1qB

F (<u>HINDIII</u>): GGG<u>AAGCTT</u>CAAAGCAGCTGCACCGGGC
T_m=58   GC=68%, AT=32%   bp=19

R (<u>EcoRI</u>): GGG<u>GAATTC</u>TTACGCATCCATGTCAGGGAAAAGCA

$T_m=58$ GC=46%, AT=54%   bp=26

Mouse C1qC

F (<u>HINDIII</u>): GGG<u>AAGCTT</u>AGCGCTGGCTGCTATGGGATC
$T_m=58$ GC=62%, AT=38%   bp=21

R (<u>EcoRI</u>): GGG<u>GAATTC</u>CTAGTCGGGAAACAGTAGGAAACCA
$T_m=58$ GC=48%, AT=52%   bp=25

Construction of vectors for the intracellular expression of globular head region of mouse C1q A, B and C chains as fusion proteins

The plasmid used for protein expression was pMal-c (New England Biolabs, Beverley, MA), which codes for maltose-binding protein under the P_{tac} promoter (de Boer, Comstock, 1983, PNAS). The primers used to amplify the DNA sequences of each of the globular heads were: ghA forward primer (FP) 5'-GGGGAATTCAGGGACCAGCCCCGGCC-3' and ghA reverse primer (RP) 3'-GGGAAGCTTTCAGGCCGAGGGGAAAATGAGGAAT-5'; ghB FP 5'-GGGGAATTCGGGGCTACACAGAAAGTCGCCT-3' and ghB RP 3'-GGGAAGCTTTTACGCATCCATGTCAGGGAAAAGCA-5'; ghC FP 5'-GGGGAATTCAAACAGAAGCACCAGTCGGTATTCACA-3' and ghC RP 3'-GGGAAGCTTCTAGTCGGGAAACAGTAGGAAACCA-5'. Plasmid pMal-c was double digested with *EcoRI* and *HindIII* and each PCR product was cloned as a *EcorI-HindIII* fragment. The polypeptide sequence thus expressed corresponded to residues of ghA, of ghB and of ghC. The recombinant vectors linked to MBP were named as , and , respectively.

<u>Globular head region of C1q A chain</u>

```
ATGGAGACCTCTCAGGGATGGCTGGTGGCCTGTGTGCTGACCATGACCCTAGTATGGACAGTGGCTGAAGATGTC
TGCCGAGCACCCAACGGGAAGGATGGGGCTCCAGGAAATCCTGGCCGCCCGGGGAGGCCGGGTCTCAAAGGAGAG
AGAGGGGAGCCAGGAGCTGCTGGCATCCGGACTGGTATCCGAGGTTTTAAAGGAGACCCAGGGGAATCTGGCCCC
CCTGGCAAACCTGGCAATGTGGGGCTCCCAGGTCCCAGTGGTCCCCTGGGGGACAGCGGCCCCCAAGGACTGAAG
GGCGTGAAAGGCAATCCAGGCAATATCAGGGACCAGCCCCGGCCAGCTTTCTCAGCCATTCGGCAGAACCCAATG
ACGCTTGGCAACGTGGTTATCTTTGACAAGGTCCTCACCAACCAGGAGAGTCCATACCAGAACCACACGGGTCGC
TTCATCTGTGCAGTGCCCGGCTTCTATTACTTCAACTTCCAAGTGATCTCCAAGTGGGACCTTTGTCTGTTTATC
AAGTCTTCCTCCGGGGGCCAGCCCAGGGATTCCCTGAGTTTCTCTAACACCAACAACAAGGGGCTCTTTCAGGTG
TTAGCAGGGGGCACCGTGCTTCAGCTGCGACGAGGGGACGAGGTGTGGATCGAAAAGGACCCCGCAAAGGGTCGC
ATTTACCAGGGCACTGAAGCCGACAGCATCTTCAGCGGATTCCTCATTTTCCCCTCGGCCTGA
```

<u>Key</u>

▬ - signal peptide   ▬ - collagen-like region   ▬ -globular head region

Figure 7a. Sequence of globular head region of mouse C1q A chain

Globular head region of C1q B chain

```
ATGAAGACACAGTGGGGTGAGGTCTGGACACACCTGTTACTGCTGCTTCTAGGTTTTCTCCATGTGTCCT
GGGCCCAAAGCAGCTGCACCGGGCCCCCTGGCATCCCTGGCATCCCTGGGGTCCCTGGGGTTCCTGGCTC
TGATGGCCAACCAGGCACTCCAGGGATAAAGGGGGAGAAAGGGCTCCCTGGACTGGCTGGAGACCTTGGT
GAGTTTGGAGAGAAAGGGGACCCAGGGATCCCTGGGACTCCAGGCAAAGTTGGCCCTAAGGGTCCCGTCG
GCCCTAAGGGTACTCCAGGCCCCTCTGGACCCCGCGGTCCCAAAGGCGATTCTGGGGACTACGGGGCTAC
ACAGAAAGTCGCCTTCTCTGCCCTGAGGACCATCAACAGCCCCTTGCGACCGAACCAGGTCATTCGCTTC
GAAAAGGTGATCACCAACGCGAACGAGAACTATGAGCCACGCAACGGCAAGTTCACCTGCAAGGTGCCTG
GCCTCTACTACTTCACCTATCATGCCAGCTCCCGGGGCAACCTGTGTGTGAATCTCGTTCGTGGCCGCGA
TCGGGACAGCATGCAGAAGTAGTCACCTTCTGTGACTATGCCCAGAACACCTTCCAGGTGACCACAGGT
GGGGTAGTCTTGAAGCTAGAGCAAGAGGAGGTTGTTCACCTGCAGGCCACAGACAAGAACTCCCTCCTGG
GCATTGAGGGTGCCAACAGCATCTTCACTGGCTTTCTGCTTTTCCCTGACATGGATGCGTAA
```

Key

▭ - signal peptide   ▮ - collagen-like region   ▯ -globular head region

Figure 7b. Sequence of globular head region of mouse C1q B chain

Globular head region of C1q C chain

```
ATGGTCGTTGGACCCAGTTGCCAGCCTCCATGTGGACTTTGCCTGCTGCTGCTGTTTCTTCTGGCCCTAC
CACTCAGGAGCCAGGCCAGCGCTGGCTGCTATGGGATCCCAGGGATGCCAGGCATGCCGGGGGCCCCTGG
GAAGGACGGGCATGATGGACTCCAGGGGCCCAAGGGAGAGCCAGGAATCCCAGCCGTCCCTGGGACCCGA
GGACCCAAGGGTCAGAAGGGCGAGCCTGGCATGCCTGGCCACCGTGGGAAAAATGGCCCCAGGGGGACCT
CAGGGTTGCCAGGGGACCCAGGCCCCAGGGGGCCTCCGGGGGAGCCAGGTGTGGAGGGCCGATACAAACA
GAAGCACCAGTCGGTATTCACAGTCACCCGGCAGACCACCCAGTACCCAGAGGCCAACGCCCTCGTCAGG
TTCAACTCTGTGGTCACCAACCCTCAGGGGCATTACAACCCAAGCACAGGGAAGTTCACCTGTGAAGTGC
CGGGCCTCTACTACTTCGTCTACTACACATCGCATACGGCCAACCTGTGCGTGCACCTGAACCTCAACCT
TGCCAGGGTGGCCAGCTTCTGCGACCACATGTTCAACAGCAAGCAGGTCAGCTCCGGAGGAGTCCTCCTG
CGGCTCCAGAGGGGCGATGAGGTGTGGCTATCAGTCAATGACTACAATGGCATGGTGGGCATAGAGGGCT
CCAACAGCGTCTTCTCTGGTTTCCTACTGTTTCCCGACTAG
```

Key

▭ - signal peptide   ▮ - collagen-like region   ▯ -globular head region

Figure 7c. Sequence of globular head region of mouse C1q C chain

Primers designed

Key: **F**: Forward primer; **R**: Reverse primer; **T$_m$**: Melting temperature in degree Celsius; **bp**: Base pairs; **restriction enzyme** used is underlined

Mouse ghA

F (EcoRI): GGGGAATTCAGGGACCAGCCCCGGCC

$T_m=59$ GC=82%, AT=18%   bp=17

R (<u>HINDIII</u>): GGG<u>AAGCTT</u>TCAGGCCGAGGGGAAAATGAGGAAT
$T_m=59$ GC= 52%, AT= 48%   bp=25

Mouse ghB

F (<u>EcoRI</u>): GGG<u>GAATTC</u>GGGGCTACACAGAAAGTCGCCT
$T_m=59$ GC=59%, AT=41%   bp=22

R (<u>HINDIII</u>): GGG<u>AAGCTT</u>TACGCATCCATGTCAGGGAAAAGCA
$T_m=58$ GC=46%, AT=54%   bp=26

Mouse ghC

F (EcoRI): GGG<u>GAATTC</u>AAACAGAAGCACCAGTCGGTATTCACA
$T_m=58$ GC=44%, AT=56%   bp=27

R (<u>HINDIII</u>): GGG<u>AAGCTT</u>CTAGTCGGGAAACAGTAGGAAACCA
$T_m=58$ GC=48%, AT=52%   bp=25

Oligonucleotide primers were planned to contain 20–40 nucleotides in length and ideally have a GC content of 40–60%.

Polymerase chain reaction

Polymerase chain reaction (PCR) was first performed for full-length mouse C1q A, B and C chains using cDNA extracted from mouse spleen as template. The following reaction mixture was set up:

5X NEB Phusion® buffer	10 µl
10mM dNTPs	1 µl
Template DNA (cDNA)	1 µl
NEB Phusion® DNA Polymerase	0.5 µl
10µM Forward primer	2.5 µl
10µM Reverse primer	2.5 µl
PCR grade water	32.5 µl
Total reaction volume	50 µl

A 'mastermix' was set up which contained the following (n):

5X NEB Phusion® buffer	10 µl
10mM dNTPs	1 µl
Template DNA (cDNA)	1 µl
NEB Phusion® DNA Polymerase	0.5 µl
PCR grade water	32.5 µl
Total reaction volume	45 µl

The template cDNA used was diluted 1:10 with PCR-grade water.

Thus, with 3 different forward and reverse primers required (n=3), a total 'mastermix' solution of the above was calculated to be n+1 (=4) plus a negative control was calculated as below:

5X NEB Phusion® buffer	50 µl
10mM dNTPs	5 µl
Template DNA (cDNA)	5 µl
NEB Phusion® DNA Polymerase	2.5 µl
PCR grade water	162.5 µl
Total reaction volume	225 µl

Then, to each Eppendorf tube, 45 µl of the above mixture was added. The tubes were labelled as mC1qA, mC1qB, mC1qC and negative control.

To the appropriate tubes, the required Forward and Reverse primers for mC1qA, mC1qB and mC1qC were added (none to the negative control). A PCR was then set up.

PCR conditions

The reaction mixture containing the appropriate primers were added to PCR tubes and placed in PCR machine. The lid was heated to 120°C. Followed by 30 cycles of 98°C for 30 seconds, 58°C for 30 seconds and 72°C for 10 minutes. Then, the reaction mixture was stored at 4°C. 5 µl of the reaction mixture was then pipetted and run on a 0.7% agarose gel.

Initial Denaturation	98°C	30 seconds

30 cycles	58°C	30 seconds
Final extension	72°C	10 minutes
Store	4°C	Indefinitely

Agarose gel

0.35 grams of DNA grade agarose was measured and dissolved in 50 ml of autoclaved water. The melting temperature of agarose is quite high and thus, this has to be dissolved by heating in microwave. Once it dissolves completely and begins to cool down, 5 µl of ethidium bromide is added to this mixture. Ethidium bromide intercalates with DNA strands and helps in detection of DNA by being visible under ultraviolet (UV) radiation exposure. However, ethidium bromide must be handled with gloves ONLY since it is carcinogenic. Then, this mixture is poured onto plate with comb so as to form wells. Once the mixture cools down, it hardens and the gel is ready to be used. 5 µl of PCR sample is added to 5 µl of loading dye (30% glycerol; 0.25% bromophenol blue), mixed well and loaded onto the wells. 1X TBE buffer (10.8 grams Tris/5.5 grams Borate/4ml of 0.5M EDTA) is added to fill up the electrophoresis tank. The gel is run at 80 volts for 60 minutes. The agarose gel is visualised under UV radiation.

PCR for mghA, mghB and mghC

As above, this time the PCR product of full-length mC1qA, mC1qB and mC1qC was used as template to extract the globular head regions.

A 'mastermix' of the following was set-up:

5X NEB Phusion® buffer	50 µl
10mM dNTPs	5 µl
NEB Phusion® DNA Polymerase	2.5 µl
PCR grade water	162.5 µl
Total reaction volume	220 µl

44 µl of the above mixture was added to each Eppendorf tube labelled as mghA, mghB, mghC and negative control.

To the appropriate tubes, 1 µl of respective template DNA from the earlier PCR was added along with the required Forward and Reverse primers (2.5 µl of each).

The final concentration of each primer in a reaction using Phusion DNA Polymerase® was recommended to be 0.2–1 μM, while 0.5 μM was recommended as optimal.

<u>Restriction enzyme digestion of PCR products and plasmid pMalC</u>

The PCR products as well as the plasmid were subjected to restriction enzyme digestion. The enzymes used to perform the digestion were EcoRI and HINDIII. The following reaction mixture using the appropriate buffer was set up:

Reaction 1 (PCR products)

PCR product	10 μl
EcoRI	1 μl
HINDIII	1 μl
10X NEB® Buffer 2	3 μl
PCR grade water	15 μl

Total reaction volume <u>30 μl</u>

Therefore, a 'mastermix' of the following (n=4 including negative control) was set up (n+1):

EcoRI	5 μl
HINDIII	5 μl
10X NEB® Buffer 2	15 μl
PCR grade water	75 μl

Total reaction volume <u>100 μl</u>

20 μl of this 'mastermix' was added to each Eppendorf tube labelled as mghA, mghB, mghC and negative control.

To the appropriate tubes, 10 μl of respective PCR product was added.

Reaction 2 (Plasmid-pMalC)

pMalC plasmid DNA	10 μl
EcoRI	1 μl
HINDIII	1 μl
10X NEB® Buffer 2	3 μl
PCR grade water	15 μl

Total reaction volume <u>30 µl</u>

<u>Ligation of digested PCR products and digested plasmid</u>

The PCR products and plasmid each having undergone restriction enzyme digestion is first heat-inactivated. In order to heat-inactivate EcoRI and HINDIII, the reaction tubes were placed in water bath with temperature set at 65°C for 20 minutes. After that, the individual PCR products and plasmid were subjected to ligation using the following reaction mixture:

Plasmid pMalC (digested)	1 µl (~ 15ng)
PCR product (digested)	5 µl (~ 100ng)
T4 Ligase	1 µl
10X T4 Ligase buffer	2 µl
DNA grade water	11 µl
Total reaction volume	<u>20 µl</u>

The reaction mixture was then kept overnight at 16°C. Following which these were added to *E.coli* TOP10 (Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG) competent cells and plated on Ampicillin-containing LB agar plates (explained in detail further).

Preparation of TOP10 *E.coli* competent cells

TOP10 *E.coli* competent cells (Genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697*gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) were inoculated overnight in 5 ml of LB at 37°C in shaking incubator. Next day, 250 µl from overnight inoculum were added to a new inoculation tube containing 25 ml of LB. This is then kept in 37°C shaking incubator till optical density (OD_{600}) reached 0.35 (0.3 to 0.4). Then, this mixture was centrifuged at 3000 RPM for 10 minutes. The supernatant was discarded (all procedures done under the hood). Pellet was re-suspended in 12.5ml of 0.1 M calcium chloride and placed on ice for at least 1 hour. Following this, another round of centrifugation at 300 RPM was carried out. This time the pellet was re-suspended in 2 ml of 0.1 M calcium chloride. The cells are thus, competent to take up external DNA (plasmid).

Transformation using ligated products

20 µl of ligation reaction mixture was added to 200 µl of above competent cells. This mixture was then kept over ice for at least 1 hour with intermittent inversions of tubes. Then, 2 minutes of heat shock was provided (transferring to water-bath with temperature set at 42°C. This is then transferred back onto ice for 10 minutes. Following this, 750 µl of LB was added and the tubes were place in 37°C incubator for 45 minutes. Then, 100 µl of this mixture was plated on to LB agar plates containing 100 µg/ml ampicillin and kept overnight in 37°C incubator. This is because the plasmid pMalC is resistant to ampicillin. And thus any colony growth would signify presence of pMalC plasmid.

Isolation of pure plasmid from transformed colonies

Individual colonies grown on the plate (as above) were inoculated overnight in 5 ml of LB containing 100 µg/ml of ampicillin. Next day, the inoculation tubes were centrifuged at 10000 RPM for 10 minutes. Pure plasmid was isolated using QIAGEN® Miniprep Plasmid Extraction kit. DNA was isolated and eluted as per manufacturer's instructions and run on 0.7% DNA agarose gel. The procedure for DNA isolation is as follows:

After harvesting the cells by centrifugation, supernatant is decanted and the bacterial pellet is suspended in 0.3 ml of Buffer P1 (resuspension buffer containing RNAase A). The bacterial cell pellet must be resuspended completely by using vortex or by pipetting until no cell clumps remain. 0.3 ml of Buffer P2 (contains Sodium hydroxide) is added and mixed by inverting tube 4-5 times (care must be taken not to vortex or this will lead to shearing of genomic DNA). Incubate at room temperature for 5 mins. 0.3 ml of ice cold Buffer P3 (contains acetic acid) is then added. This leads to precipitation and the precipitate contains genomic DNA, proteins and cell debris and Potassium dodecyl sulphate. The tube is then centrifuged at maximum speed for 10 mins. Supernatant should appear to be clear. Qiagen Tip® is placed on a rack and equilibrated with 1 ml Buffer QBT and allowed to flow by gravity. Supernatant from above step is then added to the column. Tip is then washed using 2 ml washing buffer (Buffer QC). DNA is then eluted by adding Buffer QF (high salt buffer). DNA is precipitated by adding 0.56 ml isopropanol. Reaction is mixed and centrifuged immediately at 10000 RPM for 10 mins. DNA pellet is washed with 1 ml of 70% ethanol and centrifuged at 10000 RPM for 10 mins. Pellet is air-dried for 5-10 mins and redissolved in suitable amount of DNAase-free water.

The concentrations of plasmid isolated were calculated using UV spectroscope/NanoDrop®. The appropriate amount of plasmid was then sent to Beckman Coulter Genomics® for Sanger® sequencing to check for presence of appropriate base pairs in the cloned plasmid.

Expression and purification of mouse ghA, ghB and ghC

Preparation of BL21(DE3) *E.coli* competent cells

Similar steps as before. This time BL21 (DE3) *E.coli* was used. Genotype: F⁻ *ompT hsd*S$_B$(r$_B^-$m$_B^-$) *gal dcm* (DE3). Preparation of competent cells is same as described earlier.

Transformation of competent cells using isolated plasmid

1 µl of isolated plasmid was added to 200 µl of BL 21 (DE3) *E.coli* competent cells. Rest of the procedure similar as described earlier. This time however, the plating was done on LB agar plates containing 100 µg/ml ampicillin.

Large scale protein expression and purification

Single colonies grown on the LB agar plates containing ampicillin were added to inoculation tubes containing 5 ml LB plus 100 µg/ml ampicillin. These were then kept in 37°C shaker incubator overnight. Next day, the entire contents were added to 1 litre of LB media containing 100 µg/ml ampicillin. The bacterial cells were grown to A$_{600}$ = 0.6 at 37°C. Then, they were induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG) for 3 hours. Before adding IPTG, 1ml of sample is taken aside as un-induced sample. After 3 hours inside the 37°C shaking incubator, the cells were centrifuged at 3000 RPM for 15 minutes at 4°C.

The cell pellet was then suspended in 50 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.2% v/v Tween 20, 1mM EGTA, 1mM EDTA and 5% v/v glycerol) containing 100 µg/ml lysozyme and 0.1 mM PMSF and incubated over ice for 1 hour. The cell lysate was then sonicated at 60 Hz for 30 seconds with an interval of 1 minute (15 cycles were done). Then this was centrifuged at 13000 X g for 15 minutes. The supernatant was collected and diluted (5-fold) using buffer I (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.2% v/v Tween 20, 1 mM EDTA, and 5% v/v glycerol) and passed through an amylose resin column (New England Biolabs, Beverley, MA).

Amylose Resin Column is an affinity matrix used in the isolation of proteins fused to MBP. It is used in a gravity flow column. The amylose resin is supplied in 20% ethanol and stored at 4°C. After use, the resin is stored in appropriate buffer with the addition of 0.02% Sodium Azide and can be regenerated for further use if required.

Earlier, 25 ml of the amylose resin column was set up and washed thoroughly with 5 volumes of autoclaved water, then with 5 volumes of 0.1% SDS and then again with 5 volumes of autoclaved water.

The column was then equilibrated by passing through 5 X bed volumes of buffer I. After passing thorough supernatant, the column was first washed with buffer I (3-fold column bed volume) and then with buffer II (buffer I without Tween 20; 5-fold column bed volume). Each fusion protein was then eluted with buffer II containing 10 mM maltose. To further

remove any minor contaminants, the fusion protein was applied to Q-Sepharose ion exchange column. The bound fraction was washed with column buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). The fusion protein was eluted using a gradient of 0.3 M to 0.8 M NaCl.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The eluted proteins were subjected to SDS-PAGE analysis. The gel consists of resolving gel and stacking gel, prepared in between two glass plates (thick and thin glass plate each). To prepare 12% resolving gel: 3.3 ml of water, 4 ml 30% acrylamide mix, 2.5 ml 1.5M Tris (pH 8.8), 0.1ml 10% SDS, 0.1 ml 10% ammonium persulfate and 0.004 ml of TEMED were used. After adding in between the glass plates, a layer of water is added on top so as to prevent entry of air and aid in polymerisation of gel. Following this, 5% stacking gel was prepared, containing 2.7 ml of water, 0.67 ml 30% acrylamide mix, 0.5 ml 1.0M Tris (pH 6.8), 0.1ml 10% SDS, 0.4 ml 10% APS and 0.004 ml TEMED. After discarding the layer of water on top of resolving gel, the stacking gel mixture is added on top of resolving gel. Soon, a set of combs is placed on top as well, so as to form wells to load samples.

The eluted protein samples are prepared by:

Adding equal volume of treatment buffer (0.5M Tris-HCl, pH 6.8, 4.4% SDS, 300mM mercaptoethanol, 10mg/ml bromophenol blue). A total of 10 µl of this mixture was loaded onto the wells. The gel tank was filled with 1X running buffer (Tris base 3.02g, Glycine 14.4g, SDS 1g). And the gel was run at 120 volts for about 90 minutes. Following which the gel was stained in Coomassie blue stain (10% v/w acetic acid, 0.006% (w/v) Coomassie Blue dye, 90% water). After staining, the gel was subjected to de-stain (30% methanol, 10% acetic acid, 60% water). Then the gel is viewed and analysed.

Binding specificities of mouse MBP-ghA, -ghB, ghC for heat-aggregated mouse IgG

MBP-ghA, -ghB, -ghC, and MBP (10, 5, 2.5, 1.25, 0.6 µg in 100 µl per well in carbonate buffer (pH 9.6) were coated to the microtiter wells overnight at 4°C. The wells were blocked with 2% BSA in PBS for 2 hours. After washing the wells, 10 µg/ml of heat-aggregated IgG (concentration of 10 mg/ml) in PBS with 10 mM calcium chloride were added. After incubation for 2 hours, Protein A-HRP (1:5000) was added to each well. After 1 hour, colour was developed using Sigma-OPD tablets and read at A_{405}.

Globular heads inhibitory hemolytic assays

For the CP hemolytic assay (CH_{50}):

Dextrose gelatin Veronal buffer (DGVB^{2+})

0.15 mM $CaCl_2$
141mM NaCl
0.5 mM $MgCl_2$
1.8 mM Sodium Barbitone
3.1 mM Barbitone
0.1% w/v gelatin; pH 7.4

Dulbecco phosphate buffer solution (DPBS)

0.9 mM $CaCl_2$
2.7 mM KCl
0.5 mM $MgCl_2.2H_2O$
138 mM NaCl
8.1 mM Sodium hypophosphate; pH 7.4

The above solutions were prepared.

Sheep blood (5 ml) was washed twice with DGVB^{2+} (in 10 times the volume). And then resuspended and adjusted to a concentration of 10^9 cells (OD$_{541}$ of 0.7). The OD is measured by blanking with 2.8 ml distilled water and reading lysis of sheep red blood cells (SRBC) caused by addition of 200 μl of sheep blood. This is then sensitised with anti-SRBC antibodies (Hemolysin$^®$ *Sigma*$^®$ S1389) and after 2-3 gentle inversions of the tube. This is then kept for 15 minutes in 37 degrees incubator and then 15 minutes over ice (now called EA cells). Then the tube is washed twice with DGVB; thrice with DPBS and eventually resuspended in 10 ml of DGVB^{2+}. 10^8 EA cells were used for experiments. Serial dilutions of serum (100 μl reaction volume) were made by diluting with DGVB^{2+} (1:10 to 1:1280). To each of these, 100 μl of EA cells added. 100 % lysis tube contained 100 μl distilled water + 100 μl EA cells. The tubes were incubated at 37 degrees for 1 hour and centrifuged at 7000 RPM for 10 minutes. 140 μl of supernatant was collected and read at OD$_{415}$ in ELISA plate reader.

Purification of IgG isotypes

Normal mouse serum was passed through a Protein G-sepharose (HiTrap) column. This binds IgG to the column. IgG was then eluted with glycine (ph 2.7) and collected in Eppendorf tubes. Then immediately, neutralised by adding Tris-HCl (pH 8.0). In order to purify the sub-classes of IgG, the purified IgG was passed through a Protein A-sepharose column. The flow-through contains IgG$_3$ which does not bind to Protein A. The rest of the sub-classes can be eluted at different pH of glycine according to Nikolayenko et al., (2005) (IgG$_1$ at pH 4.0, IgG$_2$ at pH 5.0 and IgG$_4$ at pH 4.5).

Binding specificities of MBP- ghA, -ghB, -ghC with IgG isotypes

1 μg/well of heat-aggregated IgG isotypes (concentration of 10 mg/ml) in PBS in 100 μl per well in carbonate buffer (pH 9.6) were coated to the microtiter wells overnight at 4°C. The wells were blocked with 2% BSA in PBS for 2 hours. After washing the wells, 10, 5, 2.5, 1.25 μg of MBP-ghA, -ghB, -ghC, and MBP in PBS with 10 mM calcium chloride were added. After incubation for 2 hours, antibody against MBP (1:5000) was added and incubated for 1 hour. Then, IgG conjugated to HRP (1:5000) was added to each well. After 1 hour, colour was developed using Sigma-OPD tablets and read at A_{405}.

Binding specificities of MBP- ghA, -ghB, -ghC with IgM

1 μg/well of heat-aggregated IgM (BD Pharmingen™ 557275) (concentration of 10 mg/ml) in PBS in 100 μl per well in carbonate buffer (pH 9.6) were coated to the microtiter wells overnight at 4°C. The wells were blocked with 2% BSA in PBS for 2 hours. After washing the wells, 10, 5, 2.5, 1.25 μg of MBP-ghA, -ghB, -ghC, and MBP in PBS with 10 mM calcium chloride were added. After incubation for 2 hours, antibody against MBP (1:5000) was added and incubated for 1 hour. Then, IgG conjugated to HRP (1:5000) was added to each well. After 1 hour, colour was developed using Sigma-OPD tablets and read at A_{405}.

Binding specificities of globular heads of mouse C1q with mouse PrP peptide

Recombinant mouse PrP was generated in *E. coli* as described in Kirby et al., (2003). Varying concentrations of mouse PrP peptide (20 μg, 10 μg, 5 μg, 2.5 μg, 1.25 μg, 0.62 μg, 0.31 μg, 0.15 μg) were coated per well overnight onto microtiter plate wells in carbonate buffer (pH 9.6) and kept at 4°C. The wells were blocked with 2% BSA in PBS for 2 hours. After washing the wells, 5 μg/ml of mghA, mghB, mghC and MBP (as negative control) in PBS with 10 mM calcium chloride were added. After incubation for 2 hours, anti-MBP (1:5000) was added to each well and kept at 37°C. After 1 hour, anti-IgG conjugated to HRP was added and kept in 37°C incubator for 1 hour. Then, colour was developed using Sigma-OPD tablets and read at A_{405}.

Binding specificities of globular heads of mouse C1q with mouse PTX3

Equal concentrations of mouse PTX3 (5 μg) was coated to each microtiter plate well in carbonate buffer (pH 9.6) and kept at 4°C. The wells were blocked with 2% BSA in PBS for 2 hours. After washing the wells, different concentrations (5 μg, 2.5 μg, 1.25 μg, 0.62 μg) of mghA, mghB, mghC and MBP (as negative control) in PBS with 10 mM calcium chloride were added. After incubation for 2 hours, anti-MBP (1:5000) was added to each well and kept at 37°C. After 1 hour, anti-IgG conjugated to HRP was added and kept in 37°C incubator for 1 hour. Then, colour was developed using Sigma-OPD tablets and read at A_{405}.

Results

Polymerase Chain Reaction

○ **Globular heads of mouse C1q A, B and C chains**
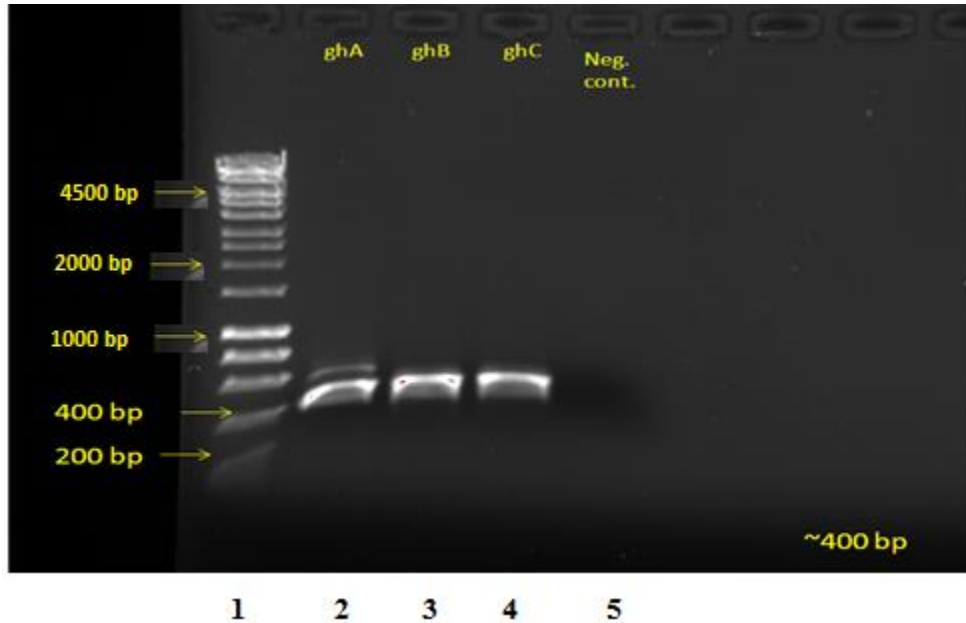
Figure 8: DNA agarose gel (0.7%) showing PCR products of globular heads of mouse C1q A, B, C chains. Lane 1: Marker ladder (Thermo Fisher Scientific© SM0633); Lane 2: mghA; Lane 3: mghB; Lane 4: mghC; Lane 5: negative (water) control. The genes were calculated to be approximately 400 bp.

Following RNA extraction and cDNA synthesis from mouse spleen, using appropriate primers, full-length mouse C1q A, B and C chains were amplified first. Following this, using the appropriate full length C1q chains, globular heads of mouse C1q A, B and C chains were amplified (mghA, mghB and mghC respectively). The gene for full-length mouse C1q A, B and C chains was calculated to be approximately 669 base pairs (bp), 684 bp and 651 bp respectively. Using this, the globular heads of mouse C1q chains namely ghA, ghB and ghC chains were generated and the genes were calculated to be approximately 400 bp.

○ **Full-length mouse C1q A, B and C chains**
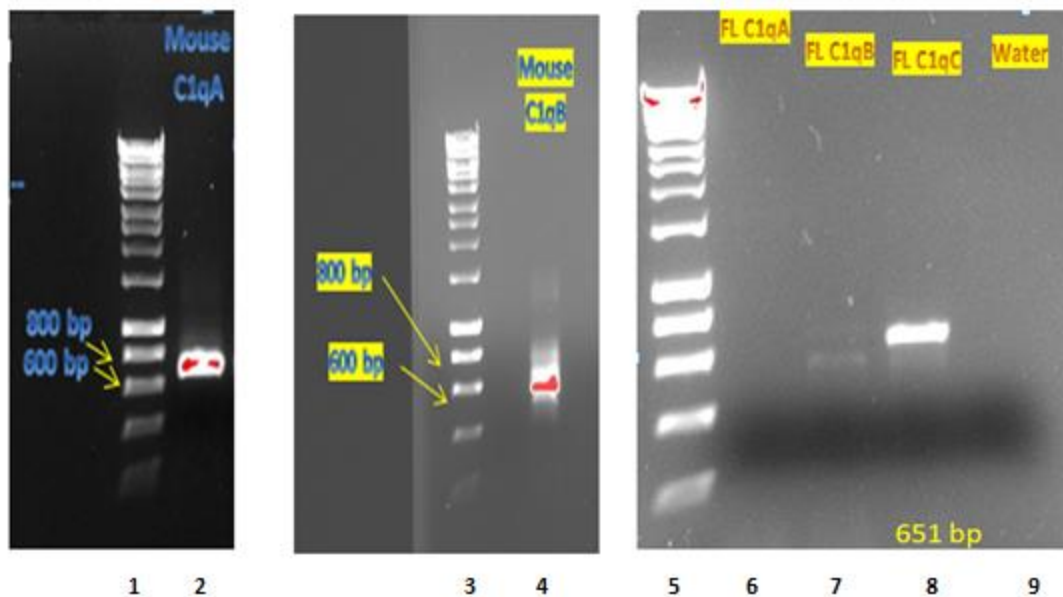
Figure 9: PCR products are visualised on DNA agarose gels (0.7%). PCR products for the mouse globular heads of C1q A, B and C chains as well as full length mouse C1q A, B and C chains are shown and labelled accordingly. Lane 1: Marker ladder; Lane 2: C1qA chain 669 bp; Lane 3: Marker ladder; Lane 4: C1qB chain 684 bp; Lane 5: marker ladder; Lane 6: inferior cloning attempt for C1qA chain (not used for ligation); Lane 7: inferior cloning attempt for C1qB chain (not used for ligation); Lane 8: C1qC chain 651 bp; Lane 9: negative (water) control; bp: base pairs).

The recombinant full length and globular heads of mouse C1q A, B and C chains were subjected to double digestion using the enzymes EcoRI and HINDIII. The 'dropped-out' segments co-related correctly to the appropriate estimated base pairs sizes. The gene cloned into the pMalc vector can be seen at approximately 4500 bp and when subjected to double digestion using the above mentioned enzymes, the C1q B and C chains can be seen at approximately 650 bp.

Double digestion of ligated products

Figure 10: Double digestion of ligated products of full length recombinant C1q B and C chains is shown. The ligated products were digested using enzymes EcoRI and HINDIII. Lane 1: Marker ladder; Lane 2-5: Full length C1qB chain 'dropping' out from the vector pMalC; Lane 6-9: Full length C1qC chain 'dropping' out from the vector pMalC.
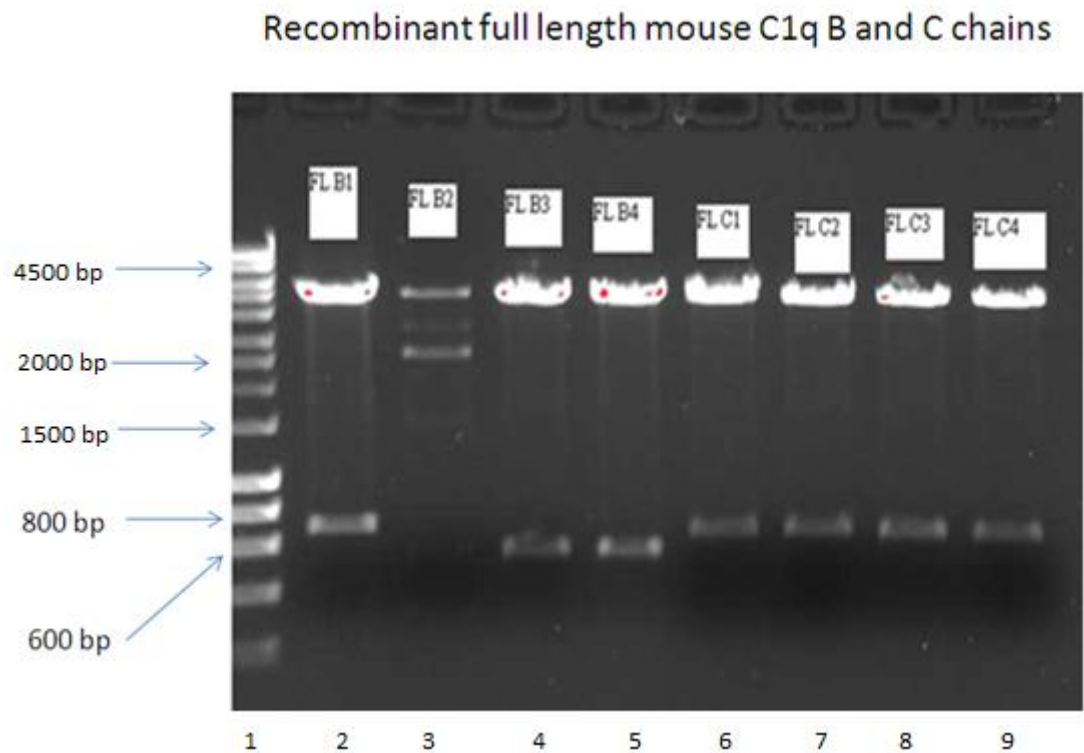
The ghA, ghB and ghC modules of mouse C1q can be expressed as soluble fusion proteins

The globular head regions of mouse C1q A, B, and C chains were expressed as proteins fused to MBP in E. coli BL21(DE3) cells. After induction with 0.4 mM isopropyl β-D-thiogalactoside for 3 hours, each fusion protein was expressed with a protein band of about 60 kDa (SDS-PAGE, reducing conditions). Most of the fusion protein bound to affinity column and eluted as pure soluble fractions. For further purification, Q-Sepharose anion-exchange column was used: Column was washed with autoclaved water X 5 bed volumes. Then washed with 1M NaCl to remove off any contaminants. The fusion proteins were passed through the column. The flow-through was collected in case no binding occurs. Different gradients of NaCl molarity were used (ranging from 0.1 M to 1 M) and the fusion proteins bound at 0.1 M NaCl while peak fractions were eluted at 0.3-0.4 M NaCl.
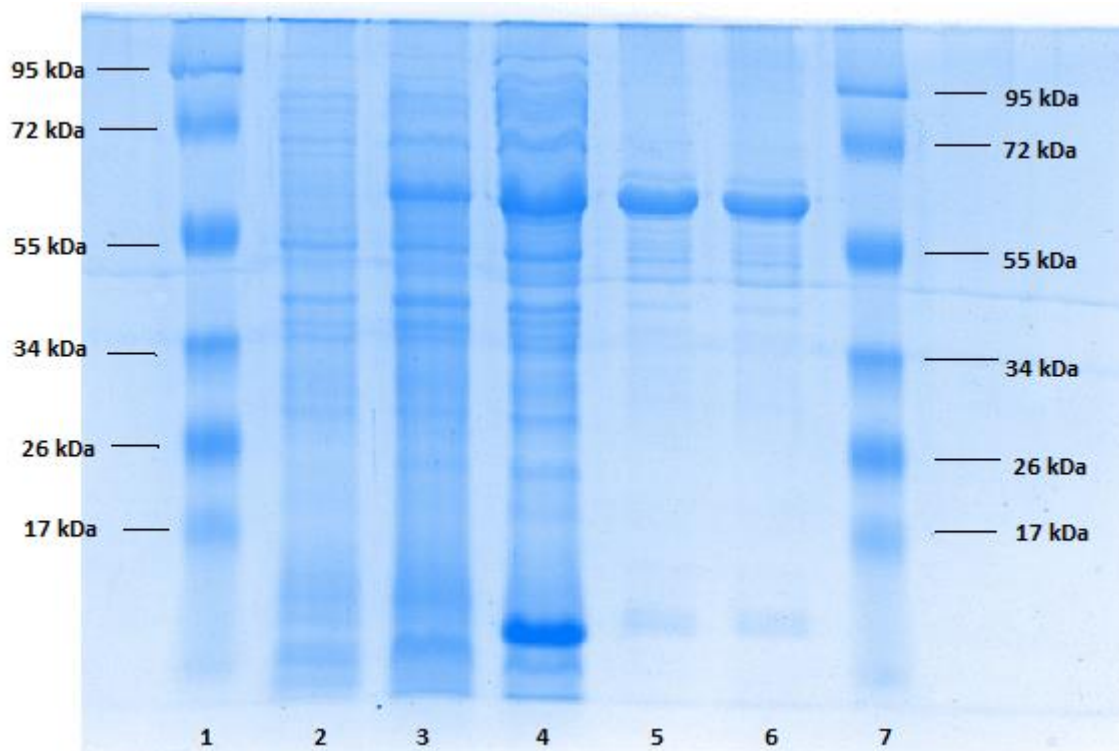
Figure 11: SDS-PAGE (12% w/v, under reducing conditions) analysis of fusion proteins MBP-ghB. *Lanes 1* and *7*, standard protein molecular mass markers; *lane 2*, uninduced MBP-ghB; *lane 3*, induced MBP-ghB; *lane 4*, after sonication MBP-ghB sample; *lane 5*, purified MBP-ghB; *lane 6*, after ion-exchange MBP-ghB.
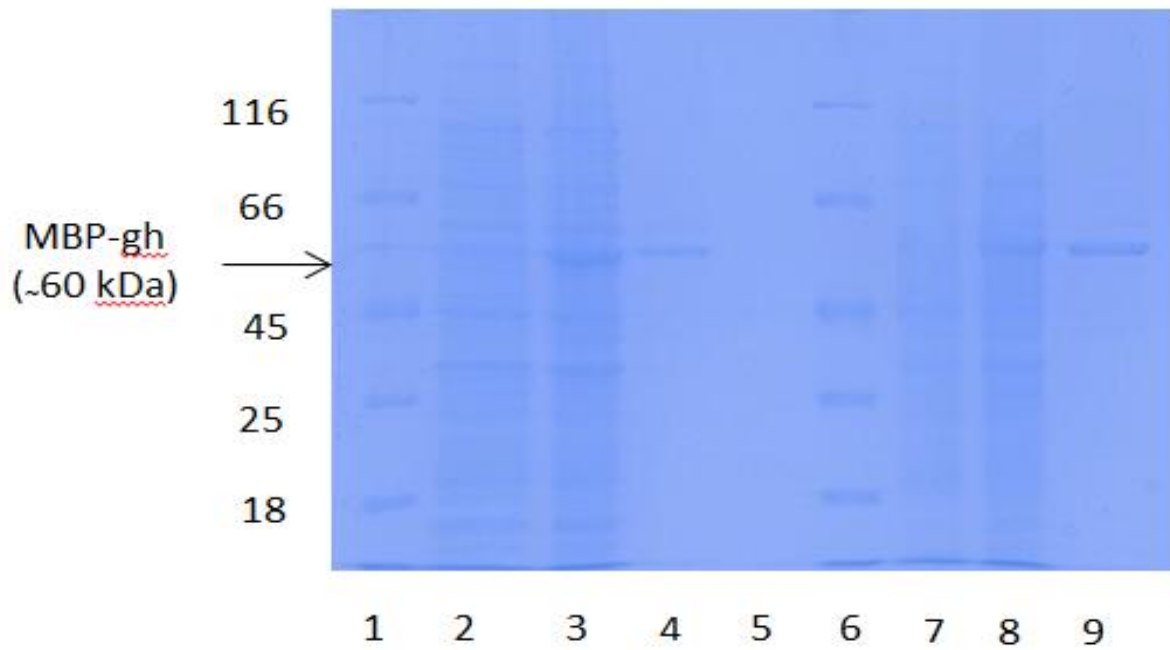
Figure 12: SDS-PAGE (12% w/v, under reducing conditions) analysis of fusion proteins MBP-ghA and MBP-ghC. *Lanes 1* and *6*, standard protein molecular mass markers; *lane 2*, uninduced MBP-ghA; *lane 3*, induced MBP-ghA; *lane 4*, purified MBP-ghA; *lane 5*, blank; *lane 7*, uninduced MBP-ghC; *lane 8*, induced MBP-ghC; *lane 9*, purified MBP-ghC.

The eluted proteins were run on SDS-PAGE (12% w/v, under reducing conditions) and analysed for presence of proteins as well as purity of proteins.

Factor Xa cleaved MBP-ghA, -ghB, -ghC
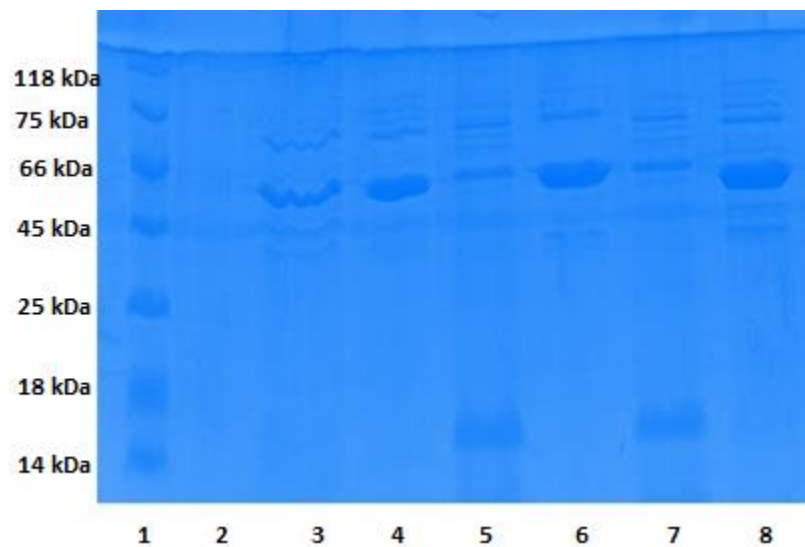
Figure 13: Factor Xa cleavage of MBP-mghA; -mghB; -mghC. The 'dropped' out fragment represents the mouse globular head of C1q after being cleaved off from MBP. Lane 1: standard protein molecular mass marker; Lane 2: blank; Lane 3: MBP-mghA; Lane 4: MBP-mghA subjected to FXa treatment; Lane 5: MBP-mghB; Lane 6: MBP-mghB subjected to FXa treatment; Lane 7: MBP-mghC; Lane 8: MBP-mghC subjected to FXa treatment.

The purified proteins were subjected to factor Xa (protease) treatment and observed for cleavage of MBP from the globular heads of C1q A, B and C chains (mghA, mghB and mghC respectively).

100 μg of respective protein fused to MBP (MBP-ghA,-ghB,-ghC) was added to Eppendorf tube. 1 μl of 1mg/ml concentration Factor Xa protease was added to each tube. Then, placed overnight at room temperature. Next day, the samples were run on SDS-PAGE (12% w/v, under reducing conditions) and analysed for presence separation of MBP from the globular heads. The separation of MBP from globular heads was confirmed in most samples although the amount and quality of cleavage varied due to probable presence of contaminants and/or concentration of Factor Xa and MBP fusion proteins.

IgG isotypes
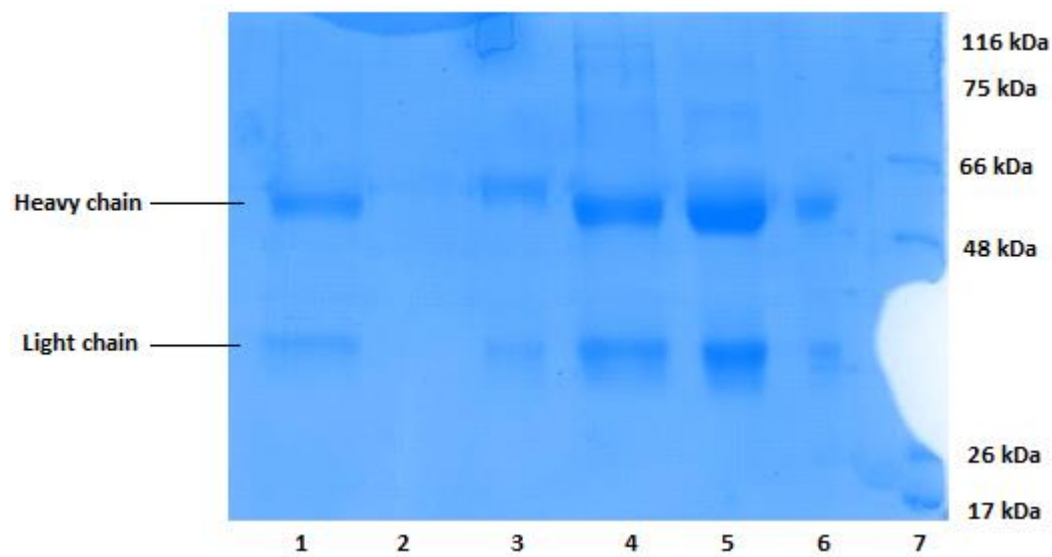
Figure 14: SDS-PAGE (10% w/v, under reducing conditions) analysis of purified IgG and IgG isotypes. Lane 1: IgG4; Lane 2: blank; Lane 3: IgG3; Lane 4: IgG2; Lane 5: IgG1; Lane 6: IgG; Lane 7: standard molecular mass protein marker.

The purified mouse IgG and its isotypes are shown on the gel. The samples were stored in -20°C upon purification. These samples were then heat-aggregated:

Samples were placed in water bath with temperature set at 65°C for 20 minutes. Then, removed and used for experiments. If not used immediately, then the samples were stored in -20°C for future use.

Binding specificities of mouse MBP-ghA, -ghB, ghC for heat-aggregated mouse IgG

Figure 15: Recombinant globular heads of mouse C1q fused to MBP (ghA-MBP, ghB-MBP, ghC-MBP) binding to heat- aggregated mouse IgG. Concentration of mouse globular heads (mghA, mghB, mghC) and MBP (negative control) are plotted on horizontal axis; Optical density at 405 nanometres is plotted along the vertical axis.

Different concentrations of mouse globular heads of C1q were coated onto wells and allowed to bind to heat-aggregated mouse IgG and then probed with HRP conjugated to Protein A. MBP-ghA, -ghB, -ghC bound to heat-aggregated IgG in a dose-dependent manner. MBP-ghA and MBP-ghB bound higher than MBP-ghC and MBP alone. It is worthwhile to note that the native C1q has 18 globular heads (which are clustered) and the recombinant ghA, ghB, and ghC are single polypeptide chains, it is difficult to compare such interactions with respect to multiple heads of C1q vs single heads of recombinant modules.

Binding specificities of mouse MBP-ghA, -ghB, ghC for heat-aggregated mouse IgM

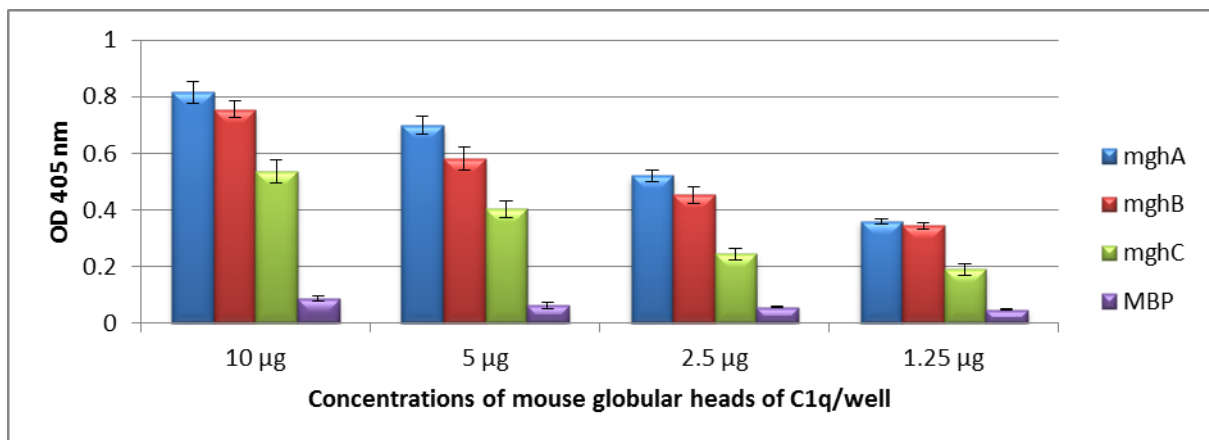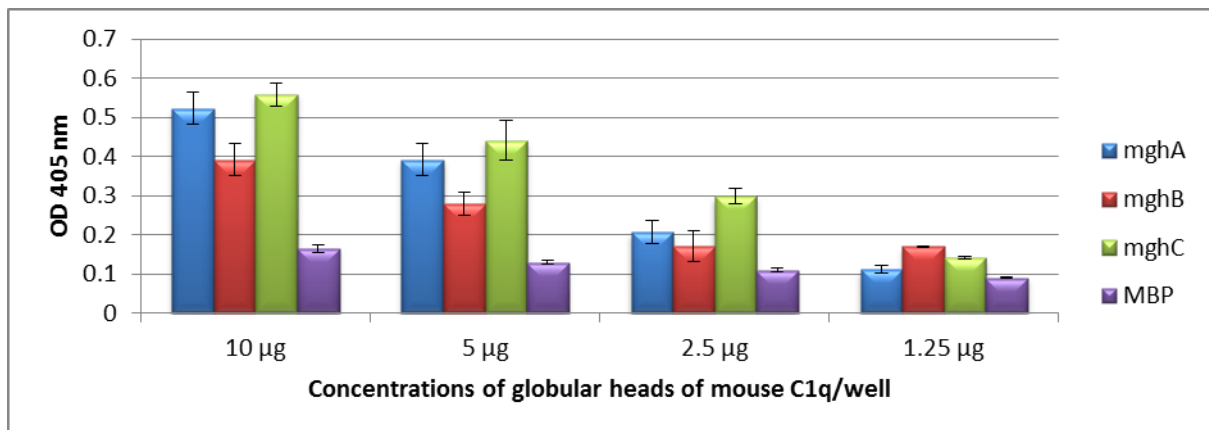**Figure 16:** Recombinant globular heads of mouse C1q fused to MBP (ghA-MBP, ghB-MBP, ghC-MBP) binding to heat- aggregated mouse IgM. Concentration of mouse globular heads (mghA, mghB, mghC) and MBP (negative control) are plotted on horizontal axis; Optical density at 405 nanometres is plotted along the vertical axis.

Different concentrations of mouse globular heads of C1q were coated onto wells and allowed to bind to heat-aggregated mouse IgM. MBP-ghA, -ghB, -ghC bound to heat-aggregated IgM in a dose-dependent manner. MBP-ghA and MBP-ghC bound higher than MBP-ghB and MBP alone. Again it is worth mentioning that the native C1q has 18 globular heads (which are clustered) and the recombinant ghA, ghB, and ghC are single polypeptide chains, it is difficult to compare such interactions with respect to multiple heads of C1q vs single heads of recombinant modules. These results indicate that the interaction of C1q with IgM might require multivalent involvement of ghA, -B and –C chains.

Globular heads inhibitory haemolytic assays
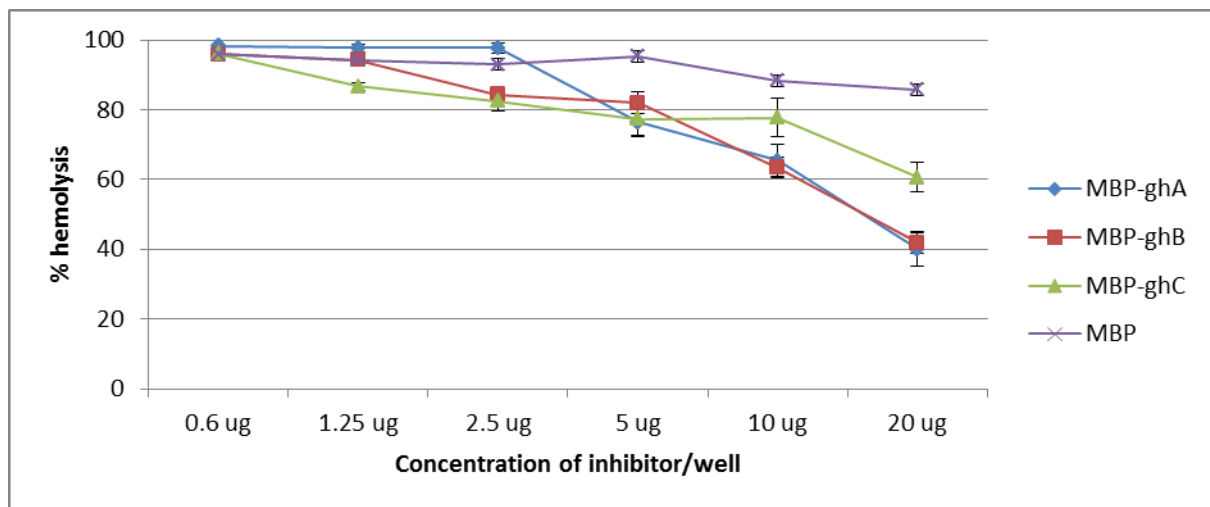
Figure 17: Globular heads inhibitory haemolytic assay. Concentration of mouse globular heads (mghA, mghB, mghC) and MBP (negative control) are plotted on horizontal axis; Percentage haemolysis observed after calculation is plotted along the vertical axis.

To examine the inhibitory effects of MBP-ghA, -ghB, and -ghC on C1q-dependent hemolysis, SRBC (E) were sensitized with hemolysin to yield EA cells. MBP-ghA, -ghB, -ghC possess the ability to inhibit C1q-dependent hemolysis of sheep erythrocytes coated with hemolysin (anti-sheep erythrocyte antibody). To achieve >50% inhibition, nearly 10 ug of MBP-ghA and 10 ug of MBP-ghB were required whereas MBP-ghC did not achieve 50% inhibition. The potencies of the three recombinant proteins as inhibitors of C1q-mediated hemolysis of sheep erythrocytes are in the order ghA nearly equal to ghB > ghC. MBP did not interfere significantly with C1q-mediated hemolysis.

Binding specificities of native human C1q and mouse MBP- ghA, -ghB, -ghC with IgG isotypes
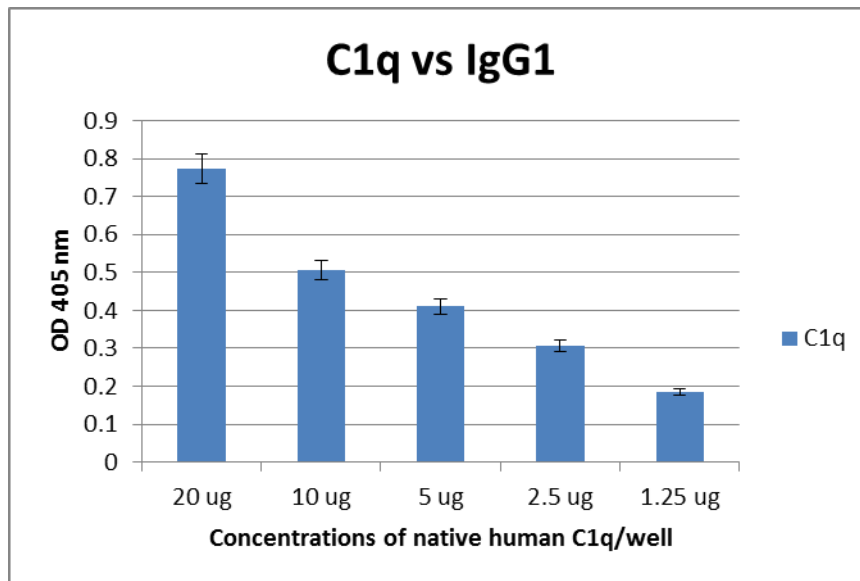
Figure 18: Binding of native human C1q with native mouse IgG1. Concentration of native human C1q per well is plotted along the horizontal axis; Optical density at 405 nanometres is plotted along the vertical axis.

Equal concentration of mouse IgG1 (10 µg/well) were coated onto wells and different concentrations of native human C1q was added. There was a dose-dependent binding between human C1q and the purified IgG1.
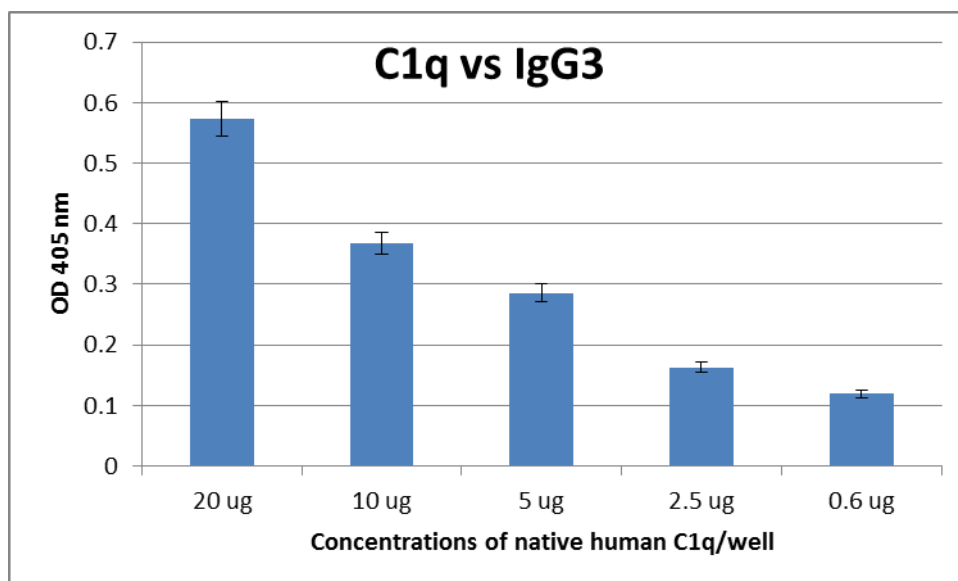
Figure 19: Binding of native human C1q with native mouse IgG3. Concentration of native human C1q per well is plotted along the horizontal axis; Optical density at 405 nanometres is plotted along the vertical axis.

Equal concentration of mouse IgG3 (10 µg/well) were coated onto wells and different concentrations of native human C1q was added. There was a dose-dependent binding between human C1q and the purified IgG3.

Binding specificities of mouse IgG1 with mouse MBP- ghA, -ghB, -ghC
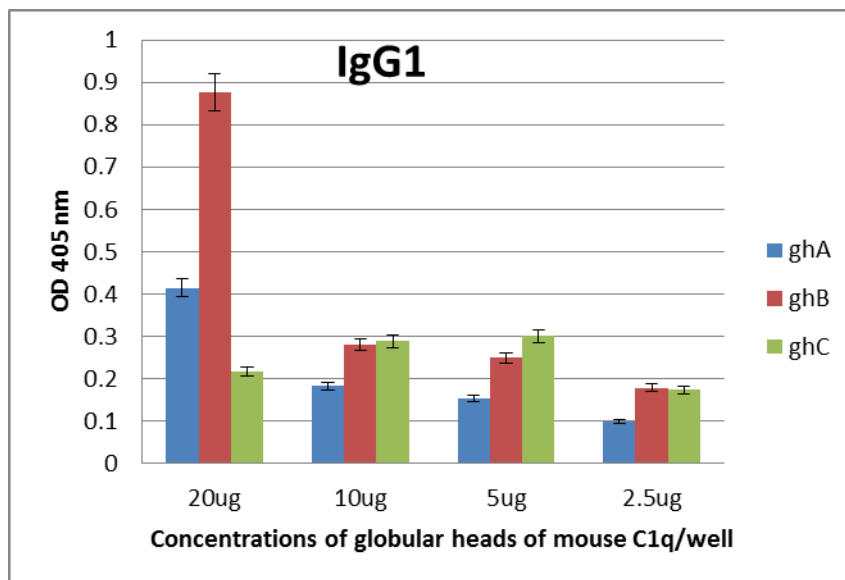
Figure 20: Binding of globular heads of mouse C1q with native mouse IgG1. Concentrations of mouse globular heads (mghA, mghB, mghC) are plotted on horizontal axis; Optical density at 405 nanometres is plotted along the vertical axis.

Equal concentration of mouse IgG1 (10 µg/well) were coated onto wells and different concentrations of globular heads of mouse C1q were added. mghA, mghB and mghC bound to IgG1 in a dose-dependent manner. MBP-ghB showed highest binding at 20 µg concentration as compared to mghA, mghC.

Binding specificities of mouse globular heads of mouse C1q with mouse prion protein peptide
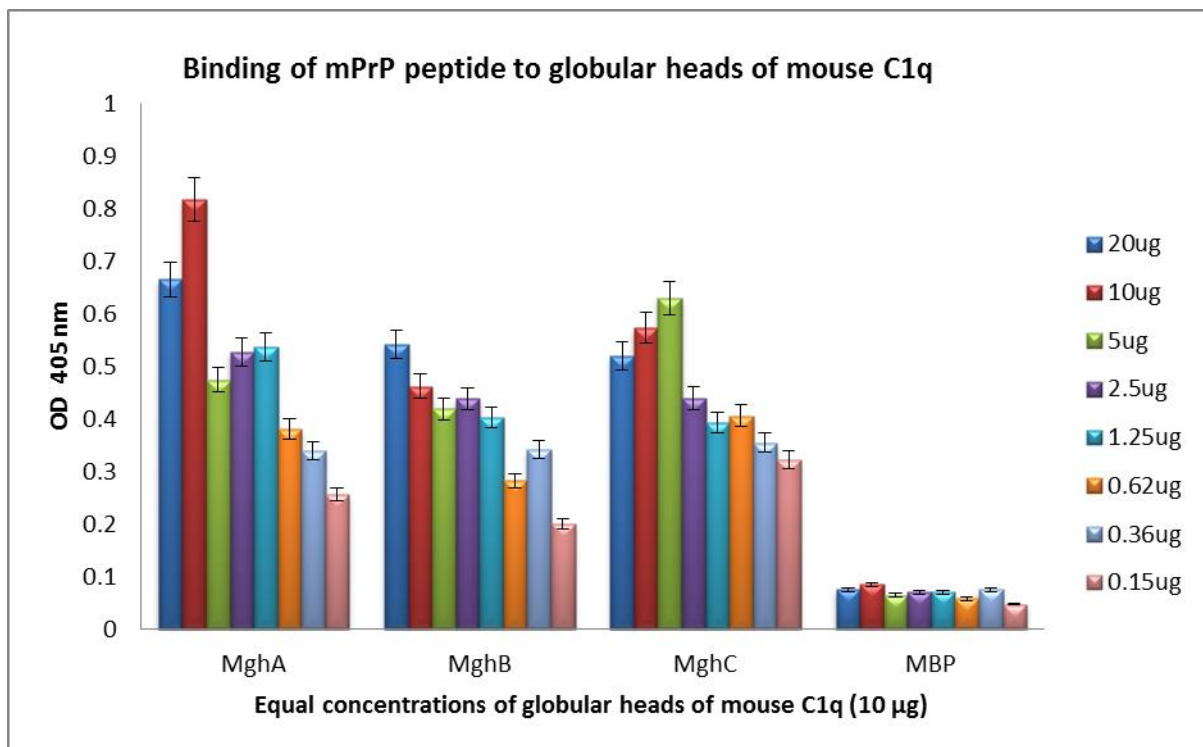
Figure 21: Binding of globular heads of mouse C1q with recombinant mouse PrP peptide. Equal concentration (5 μg) of mouse globular heads (mghA, mghB, mghC) and MBP (negative control) are plotted on horizontal axis; Optical density at 405 nanometres is plotted along the vertical axis.

Reducing concentrations of mouse PrP peptide was coated on to wells and equal concentration (5 μg) of ghA, ghB, ghC and MBP as negative control was added. Highest binding strength was observed for ghA at 10 μg concentration and a relative dose dependency was observed. MBP as negative control did not appear to interfere significantly with the binding process.

Binding specificities of globular heads of mouse C1q with mouse PTX3
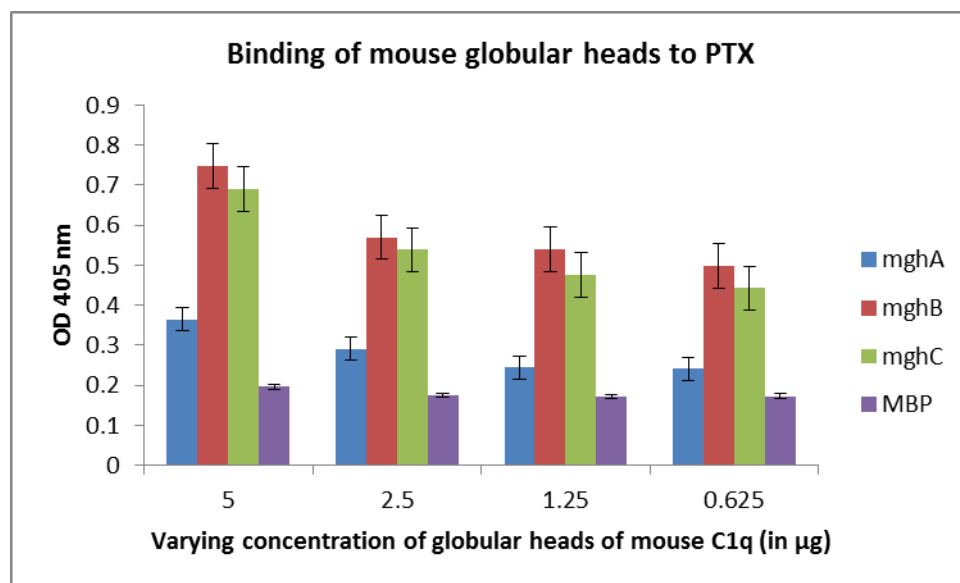
Figure 22: Binding of recombinant globular heads of C1q with PTX3. The recombinant globular heads of mouse C1q fused to MBP (ghA-MBP, ghB-MBP, ghC-MBP) binding to heat- aggregated mouse IgG. Concentration of mouse globular heads (mghA, mghB, mghC) and MBP (negative control) are plotted on horizontal axis; Optical density at 405 nanometres is plotted along the vertical axis.

Equal concentration of mouse PTX3 (10 μg) was coated on to wells and reducing concentrations of ghA, ghB, ghC and MBP were added (5 μg, 2.5 μg, 1.25 μg, 0.625 μg). ghB and ghC modules were seen to bind well with PTX3 as compared to ghA. Dose dependency was observed. MBP as negative control did not significantly interfere with binding process.

Discussion

C1q is known to interact with a range of know ligands via its gC1q domain. The expression and functional characterisation of individual mouse gC1q domains has allowed to observe for specificity and selectivity of individual globular heads of muse C1q to known ligands. Binding specificities of gC1q to IgG as well as IgG isotypes has been studied. It is also seen that ghB is inhibitory in the haemolytic assays.

The generation of recombinant forms of ghA, ghB, ghC will help in better understanding of their binding abilities either autonomously or collectively. Also, such studies have not been conducted in mouse C1q. This will enable better understanding of mouse system of functioning and also help in understanding pathogenesis of diseases in mouse models of disease. Future work will involve binding studies involving mouse prion protein. It will be interesting to see which globular head binds preferentially to prion protein and also to study pathogenesis mechanisms of prion disease. Production of recombinant full-length C1q will be the next step. Also structural alignment of mouse gC1q in relation to human gC1q will be conducted.

MBP-ghA, -ghB, -ghC bound to heat-aggregated IgG in a dose-dependent manner. MBP-ghA and MBP-ghB bound higher than MBP-ghC and MBP alone. Studies have shown that the individual modules of gC1q bind to their ligands differentially. Heat-aggregated IgG binds preferentially to ghB and ghA while heat-aggregated IgM shows preferential binding to ghC (Kishore et al., 2003). C1q is known to bind via its gC1q domain to non-aggregated IgG at the Fc region weakly (Duncan and Winter, 1988; Hughes-Jones and Gardner, 1979), but the strength of binding increases nearly a thousand-fold when immune complexes (or heat-aggregation) of IgG are involved (Kishore et al., 2002) The binding site has been localised to CH2 domain of the Fc portion of IgG with the residues Glu (E)-318, Lys (K)-320 and Lys (K)-322 being significant in this binding (Duncan and Winter, 1988). Binding site of C1q is different in human and mouse IgG sub-types (Idusogie et al., 2001; Idusogie et al., 2000; Thommesen et al., 2000) and so are the binding affinities and complement-activating functions (Leatherbarrow and Dwek, 1984; Bindon et al., 1988). Interaction of C1q with CRP is known to occur via the gC1q region (Agrawal et al., 2001). Activation of acute phase protein, CRP leads to beneficial anti-inflammatory host defence system. PTX3 is structurally similar to CRP and also binds C1q via the gC1q region (Nauta et al., 2003).

MBP-ghA, -ghB, -ghC bound to heat-aggregated IgM in a dose-dependent manner. MBP-ghA and MBP-ghC bound higher than MBP-ghB and MBP alone. It is known for earlier studies that the interaction of C1q with most of the negatively charged ligands includes an initial step of binding to residues on gC1q apex, followed by rotation of gC1q facilitated by Ca^{2+} and then binding by the residues on ghB or C1qB chain. The three residues Lys^{A200} from A chain, Tyr^{B175} from B chain and Lys^{C170} from C chain, all from apex of gC1q region are crucial and take part in the interaction with immunoglobulins (Gadjeva et al., 2008).

Both MBP-ghA and MBP-ghB also inhibited C1q-dependent hemolysis of IgG sensitized sheep erythrocytes. The specificity and selectivity gives a high potential of flexibility and versatility to C1q for interaction with ligands. However it is worth noting that the full intact C1q molecule has 6xC1qA, 6xC1qB and 6xC1qC chains and the studies conducted here involve on individual ghA,-B, and –C region (Kishore et al., 2003).

Binding of prion peptide with gC1q region would help in understanding the pathogenesis of prion disease. Currently, it is understood that PrP^{Sc} in the gut activates the complement system mainly by C1q and leads to it being opsonised and undergoes uptake by dendritic cells. The dendritic cells lead to its transportation into the lymphatic system wherein the follicular dendritic cells facilitate replication and further involvement of CNS. PrP has been found to be capable of activating complement in an antibody-independent manner (Mitchell et al., 2007). Earlier studies have shown the importance of Ca^{2+} in the binding between C1q and PrP with Ca^{2+} leading to a conformational change in the globular region of prion protein leading to C1q binding (Blanquet-Grossard et al., 2005).

The study of recombinant gC1q region heads will help in understanding the effects of PTX3 activation on the complement system. It is known that binding of C1q via gC1q region to PTX3 is highly electrostatic in nature as the interaction *in vitro* is highly dependent on the ionic strength of binding buffers. The binding is also known to be pH-dependent thereby implying the importance of inflammatory site pH in the body. And as mentioned previously, the residues (Tyr^{B175} and Lys^{C170}) from the apex and side surface of ghB are vital in the interaction with PTX3 (Roumenina et al, 2006).

C1q is involved in development of SLE. Immune complexes trigger the complement pathway and C1q is thought to play a crucial role in tissue injury. However, congenital complete or partial deficiency of C1q also leads to SLE. And C1q is also involved in apoptotic cell clearance as well as immunoregulation of B cells and T cells providing indication of beneficial role in SLE (Sontheimer et al., 2005).

The anti-C1q antibodies generated in human body are also directed against the gC1q region namely against gh-A, -B and –C regions. The anti-C1q autoantibodies against ghB have been shown to inhibit binding of C1q to IgG and CRP leading to inefficient apoptotic cell and immune complex clearance as well as leading to generation of pro-inflammatory cytokines (Radanova et al., 2012; Tsacheva et al., 2007). Production of mouse gC1q domains will be helpful in antibody mapping of anti-C1q autoantibodies recognising gC1q region.

The production of individual gC1q domains also open up the possibilities for mutational analysis studies, helping in assessment of residues and motifs involved in C1q interaction with its ligands.

C1q-deficient mice ($C1qa^{-/-}$) are highly susceptible to development of SLE caused by impairment in clearance of apoptotic cells. These C1q knock-out mice develop antinuclear

antibodies and glomerulonephritis (associated with multiple apoptotic bodies). C1q has also been found to be involved in apoptotic cell clearance without the need for C3 activation (Botto et al., 1998; Mitchell et al., 1999). Antibodies can be raised against the individual globular heads of mouse gC1q region and can be integrated with C1q knock-out mouse model of disease for study of involvement of individual globular heads or antibodies against individual gC1q heads in disease pathogenesis. Future work involving production of full-length recombinant mouse C1q can again be tested in C1q-deficient mice to test for efficiency and probable role in disease therapeutics as well as characterisation of *in vivo* functioning of recombinant mouse C1q.

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Appendix 1: Alignment of mouse gC1q with human gC1q
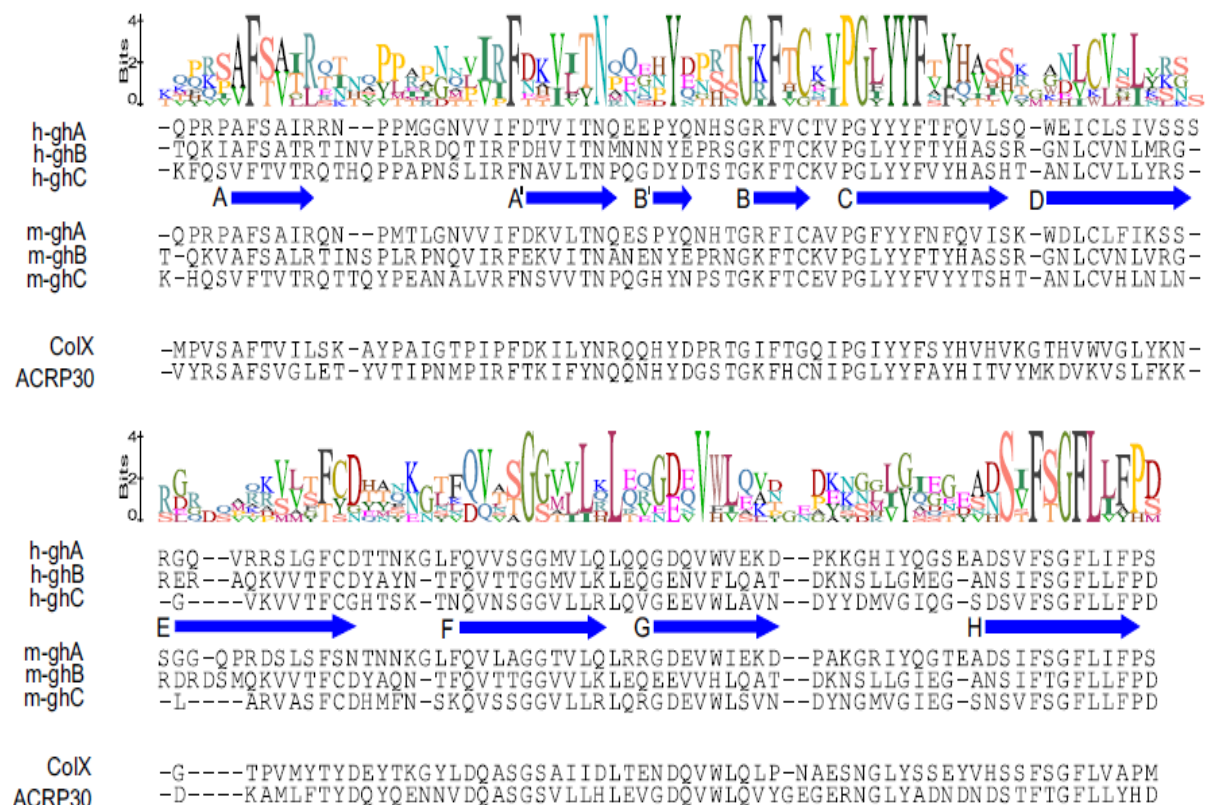
Alignment of the mouse gC1q domains with the human gC1q domains, Collagen X and ARCP

Appendix 2: Details of protein sequence of mouse gC1q

Complete mouse protein sequences (with signal peptide in bold underline. C1Q domains in BOLD

>mouse_c1qa NP_031598.2

METSQGWLVACVLTMTLVWTVAEDVCRAPNGKDGAPGNPGRPGRPGLKGERGEPGAAGIRTG
IRGFKGDPGESGPPGKPGNVGLPGPSGPLGDSGPQGLKGVKGNPGNIRDQPRP**AFSAIRQNP
MTLGNVVIFDKVLTNQESPYQNHTGRFICAVPGFYYFNFQVISKWDLCLFIKSSSGGQPRDS
LSFSNTNNKGLFQVLAGGTVLQLRRGDEVWIEKDPAKGRIYQGTEADSIFSGFLI**FPSA

>mouse_c1qb NP_033907.1

MKTQWGEVWTHLLLLLLLGFLHVSWAQSSCTGPPGIPGIPGVPGVPGSDGQPGTPGIKGEKGL
PGLAGDLGEFGEKGDPGIPGTPGKVGPKGPVGPKGTPGPSGPRGPKGDSGDYGATQKV**AFSA
LRTINSPLRPNQVIRFEKVITNANENYEPRNGKFTCKVPGLYYFTYHASSRGNLCVNLVRGR
DRDSMQKVVTFCDYAQNTFQVTTGGVVLKLEQEEVVHLQATDKNSLLGIEGANSIFTGFLL**F
PDMDA

>mouse_c1qc NP_031600.2

MVVGPSCQPPCGLCLLLLFLLALPLRSQASAGCYGIPGMPGMPGAPGKDGHDGLQGPKGEPG
IPAVPGTRGPKGQKGEPGMPGHRGKNGPRGTSGLPGDPGPRGPPGEPGVEGRYKQKHQS**VFT
VTRQTTQYPEANALVRFNSVVTNPQGHYNPSTGKFTCEVPGLYYFVYYTSHTANLCVHLNLN
LARVASFCDHMFNSKQVSSGGVLLRLQRGDEVWLSVNDYNGMVGIEGSNSVFSGFLL**FPD

mouse sequences <u>without sequence peptide</u> (**<u>all residue
numberings are according to these sequences</u>**) C1q domain in
BOLD. 4 residues in front of each and 3 residues at the back
are underlined. These were taken in addition to the domain for
modeling the domain structure (to align properly to Gerard's
structural alignment.

>mouse_c1qa NP_031598.2

METSQGWLVACVLTMTLVWTVAEDVCRAPNGKDGAPGNPGRPGRPGLKGERGEPGAAGIRTG
IRGFKGDPGESGPPGKPGNVGLPGPSGPLGDSGPQGLKGVKGNPGNIRD<u>QPRP</u>**AFSAIRQNP
MTLGNVVIFDKVLTNQESPYQNHTGRFICAVPGFYYFNFQVISKWDLCLFIKSSSGGQPRDS
LSFSNTNNKGLFQVLAGGTVLQLRRGDEVWIEKDPAKGRIYQGTEADSIFSGFLI**<u>FPSA</u>

>mouse_c1qb NP_033907.1

MKTQWGEVWTHLLLLLLLGFLHVSWAQSSCTGPPGIPGIPGVPGVPGSDGQPGTPGIKGEKGL
PGLAGDLGEFGEKGDPGIPGTPGKVGPKGPVGPKGTPGPSGPRGPKGDSGDYGA<u>TQKV</u>**AFSA

LRTINSPLRPNQVIRFEKVITNANENYEPRNGKFTCKVPGLYYFTYHASSRGNLCVNLVRGR
DRDSMQKVVTFCDYAQNTFQVTTGGVVLKLEQEEVVHLQATDKNSLLGIEGANSIFTGFLL<u>F</u>
<u>PDMDA</u>

>mouse_c1qc NP_031600.2

MVVGPSCQPPCGLCLLLLFLLALPLRSQASAGCYGIPGMPGMPGAPGKDGHDGLQGPKGEPG
IPAVPGTRGPKGQKGEPGMPGHRGKNGPRGTSGLPGDPGPRGPPGEPGVEGRYKQK<u>HQS</u>**VFT**
VTRQTTQYPEANALVRFNSVVTNPQGHYNPSTGKFTCEVPGLYYFVYYTSHTANLCVHLNLN
LARVASFCDHMFNSKQVSSGGVLLRLQRGDEVWLSVNDYNGMVGIEGSNSVFSGFLL<u>FPD</u>

here are the mouse domains extracted from the mouse c1q
proteins,

>mc1qa QPRP-4 extra residues taken in front and 3 from back
FPS

QPRP**AFSAIRQNPMTLGNVVIFDKVLTNQESPYQNHTGRFICAVPGFYYFNFQVISKWDLCL**
FIKSSSGGQPRDSLSFSNTNNKGLFQVLAGGTVLQLRRGDEVWIEKDPAKGRIYQGTEADSI
FSGFLIFPS

>mc1qb TQKV-4 extra residues taken in front and 3 from back
FPD

TQKV**AFSALRTINSPLRPNQVIRFEKVITNANENYEPRNGKFTCKVPGLYYFTYHASSRGNL**
CVNLVRGRDRDSMQKVVTFCDYAQNTFQVTTGGVVLKLEQEEVVHLQATDKNSLLGIEGANS
IFTGFLLFPD

>mc1qc KHQS-4 extra residues taken in front and 3 from back
FPD

KHQS**VFTVTRQTTQYPEANALVRFNSVVTNPQGHYNPSTGKFTCEVPGLYYFVYYTSHTANL**
CVHLNLNLARVASFCDHMFNSKQVSSGGVLLRLQRGDEVWLSVNDYNGMVGIEGSNSVFSGF
LLFPD

Appendix 3: Most variable residues in mouse gC1q

Table 3									
Most variable residues within mouse gC1q identified by ConSurf									
		m-ghA			m-ghB			m-ghC	
1		ISO[1]	98	•	THR[2]	102	•	THR[1]	101
2	•	MET[1]	103	•	ASN[1]	104	•	GLN[1]	102
3	•	THR[1]	104	•	SER[1]	105	•	TYR[1]	103
4	•	LEU[1]	105	•	PRO[1]	106	•	GLU[2]	105
5	•	GLY[1]	106	•	**ARG[1]**	108	•	ALA[1]	106
6		**LYS[2]**	113	•	PRO[1]	109	•	ALA[1]	108
7	○	SER[1]	120	•	GLN[1]	111	•	**ARG[1]**	111
8	○	PRO[1]	121		**LYS[1]**	117	•	SER[1]	114
9	•	HIS[1]	125	○	ALA[2]	122	○	PRO[1]	119
10	•	**ARG[1]**	128	○	GLU[1]	124	○	GLY[1]	121
11		ALA[1]	132		**ARG[1]**	129	•	PRO[1]	125
12		**LYS[2]**	146		**LYS[1]**	136	•	SER[1]	126
13		PHE[1]	152		LEU[1]	140		GLU[1]	133
14	○	SER[2]	157	•	ASN[1]	156		LEU[1]	137
15	○	GLY[1]	158	•	VAL[1]	158	○	ASN[1]	155
16	○	GLY[1]	159	•	**ARG[1]**	159	○	LEU[1]	158
17	○	PRO[1]	161	•	GLY[2]	160	○	ALA[1]	159
18	○	**ARG[2]**	162	•	**ARG[1]**	161	○	**ARG[1]**	160
19	○	ASP[1]	163	•	ASP[1]	162	•	PHE[1]	169
20		THR[1]	170	•	**ARG[1]**	163	•	ASN[1]	170
21		LEU[1]	175	•	MET[1]	166	○	**ARG[1]**	182
22	•	GLN[1]	186	•	GLN[1]	167	○	GLN[1]	184
23	•	**ARG[1]**	188	•	**LYS[1]**	168	○	**ARG[1]**	185
24	•	**ARG[1]**	189	○	GLN[1]	177	•	ASP[1]	195
25	○	PRO[1]	199	○	ASN[2]	178	•	TYR[1]	196
26	○	ALA[1]	200	○	THR[2]	179		GLU[1]	203
27		THR[1]	208	○	PHE[2]	180			
28				•	**LYS[2]**	190			
29				•	GLU[1]	192			
30				•	GLN[1]	193			
31				○	ISO[2]	210			
32				○	GLU[1]	211			

Residues are sorted based on their position. (•) and (○) indicate possible important residue clusters for ligand binding. Clusters are defined wherever residues are at/within 3-residue distance from each other. Hydrophobic and ring residues are underlined. Arginine and Lysine residues are shown in boldface. Numbers in superscript indicate the "rank" of the residue given by ConSurf (1-9, most variable to most conserved). Only the most variable residues (ranks 1 and 2) are shown here.