

Understanding cellular and molecular
interactions of gC1qR, a receptor for the
globular domain of complement protein C1q

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Lina Pednekar

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Abstract

gC1qR was originally discovered as a C1q receptor specific to the globular head domain of C1q, the first subcomponent of the classical pathway of complement activation. During the same period, calreticulin (CRT), formerly called as cC1qR, was described as a receptor for the collagen region of C1q and collectins. Although much work has been carried out with relation to CRT-CD91 complex, the biological implications and structure-function studies of C1q-gC1qR interaction has not been further explored. With passage of time since 1994, it has become evident that gC1qR is also a multi-functional pattern recognition receptor that can recognise pathogens in addition to acting as a modulator of inflammation at the site of injury or infection.

In this thesis, a recombinant form of gC1qR using a T7 promotor expression system was expressed and examined for its interaction with individual globular head modules of C1q A, B and C chains (ghA, ghB and ghC, respectively). A number of single residue substitution mutants of ghA, ghB and ghC modules were also analysed for their interaction with gC1qR in order to map complementary binding sites. Concomitant expression of gC1qR and C1q in the adherent monocytes with, and without proinflammatory stimuli was analysed by qPCR in order to establish autocrine/paracrine basis of C1q-gC1qR interaction. In addition, experiments were carried out to examine if C1q-mediated anti-lymphoproliferative effect can be altered by gC1qR. Subsequently, using the wild type and mutants of ghA, ghB and ghC modules, the interaction of DC-SIGN and SIGN-R, a newly discovered partner of C1q and gC1qR on the dendritic cell surface, was examined. Experiments are underway to understand how a trimolecular complex involving C1q, gC1qR and DC-SIGN participate during HIV-1 infection. Structure-function studies involving gC1qR and HCV core protein and HIV-1 gp41 have also been carried out to localise domains of gC1qR responsible for viral pathogenesis. The last chapter dwells on a newly discovered ability of gC1qR to upregulate bradykinin 1 receptor on the endothelial cell surface, thus its role in altering vascular permeability and the contact system.

The thesis describes (1) localisation of interacting sites between C1q and gC1qR and their togetherness in co-expression under pro-inflammatory conditions and possibly suppression of

immune cell proliferative response; (2) localisation of complementary binding sites between DC-SIGN, gC1qR and C1q and its possible implications in HIV-1 infection and antigen presenting cells such as dendritic cells; (3) localisation of interacting sites between gC1qR and HCV core protein as well as HIV-1 gp41 peptides with potential to propose a therapeutic peptide; and (4) ability of gC1qR to upregulate bradykinin 1 and 2 receptors on endothelial cells and its newly identified function as a modifier of inflammation.

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List of abbreviations

A-beta	Amyloid beta
AD	Alzheimer's Disease
AP	Alkaline Phosphatase
APC	Antigen Presenting cell
B1R	Bradykinin Receptor 1
B2R	Bradykinin Receptor 2
BCA	Bicinchoninic Acid
BK	Bradykinin
BSA	Bovine Serum Albumin
CLR	Collagen Like Region
CNS	Central Nervous System
CPS	Capsular pneumococcal polysaccharide
CR1	Complement Receptor 1
CRD	Carbohydrate Recognition Domain
CRP	C-Reactive Protein
CRT	Calreticulin
DAB	Diaminobenzidine peroxidase
DC	Dendritic Cells
DC-SIGN	Dendritic Cell Specific ICAM3 grabbing Non Integrin
DEAE	Diethylaminoethanol
DGVB++	Dextrose Gelatin Veronal Buffer
DPBS	Dulbecco's Phosphate Buffer
EA	Sensitised Erythrocytes
EC	Endothelial Cell
ELISA:	Enzyme Linked Immuno Sensitive Assay
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FGN	Fibrinogen
FXII	Coagulation factor XII
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
Hast v1-CP	Human Astrovirus Coat Protein
HCV	Hepatitis C Virus
HK	High Molecular Weight Kininogen
HNP-1	Human neutrophil peptide-1
HRP	Horseradish Peroxidase
HRP	Horseradish Peroxidase
ICAM:	Intracellular Adhesion Molecule
iDCs	Immature Dendritic Cells
IPTG	Isopropyl- β -D-thiogalactopyranoside
KKS	Kallikrein/Kinin system
LB	Luria Broth:

LDL	Low Density Lipoproteins
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MBP	Maltose Binding Protein
MF	Membrane Face
MZM	Marginal Zone Macrophages
NHS	Normal Human Serum
NK	Natural Killer
OPD	O-Phenylenediamine
PA	Protein A
pAb	Polyclonal Antibody
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
Pha	Phytohaemagglutinin
PK	Prekallikrein
PMC	Peritoneal mast cells
PrP	Prion Protein
PRRs	Pattern Recognition Receptors
PS	Phosphatidylcholine
PTX	Pentraxin 3
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid Arthritis
SAP	Serum Amyloid Protein
SDS	Sodium Dodecyl Sulphate
SF	Solution Face
SIGN-R	Dendritic Cell Specific ICAM3 grabbing Non Integrin Related
SLE	Systemic Lupus Erythematosus
SRBCs	Sheep Red Blood Cells
TBE	Tris Boric acid EDTA
TBS	Tris Base Saline
TCR	T-cell Receptor
Th	T-Helper
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
uPar	Urokinase-type Plasminogen Activator Receptor
WT	Wild Type

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CHAPTER 1:
INTRODUCTION

1.1 Innate and Adaptive Immune system

The immune system essentially functions to actively remove infectious non-self microbes invading the body. The immune system is split into two major components, each with its own critical function in response to infection and disease. The first is known as the innate immune system which includes sensors to detect the invading pathogen through Pattern Recognition Receptors (PRRs) expressed on innate immune cells. These PRRs recognize specific microbial motifs known as pathogen associated molecular patterns (PAMPs). This recognition leads to the secretion of various cytokines to bring upon necessary immune responses which make up the second component known as the adaptive immune system. This system is highly specific and diverse in discriminating between different PAMPs and essentially carries out specific antibody and cell mediated responses performed by B cells and T cells. The innate immune system serves as the body's first line of defence, whereas the adaptive system behaves as a secondary response system where it comes into force to eliminate pathogens that have overcome initial innate immune responses. Adaptive immunity is also 'adapted' to respond against previously encountered pathogens due to its persistent memory. In spite of these differences, adaptive immunity is brought upon by the innate immune response due to a range of cellular and humoral factors.

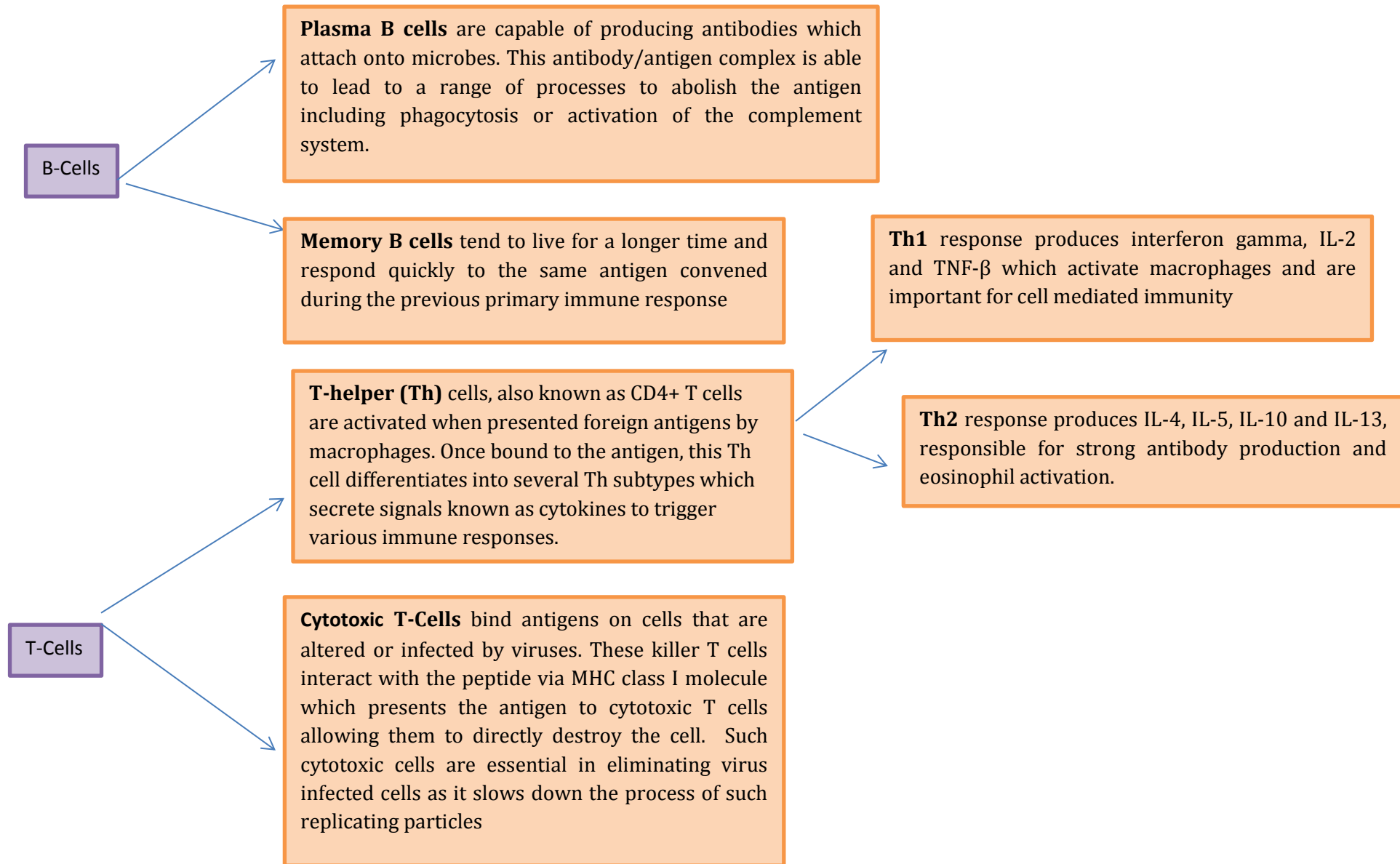
1.2 Components of Innate Immune System

Cells of the innate immune system include leucocytes which are made up of phagocytes and lymphocytes. Phagocytic cells are macrophages, neutrophils and dendritic cells (DC). Neutrophils circulate the blood stream and arrive at sites of inflammation to encounter invading microbes by a process called phagocytosis. This occurs through the plasma membrane of the neutrophil extending (known as pseudopodia) around the foreign particle to enclose it into a phagosome inside the cell. Killing of the phagosome is carried out by the neutrophil through the release of cytotoxic chemicals and enzymes. A non-mitochondrial oxidase enzyme is activated which generates toxic oxygen species (Segal and Jones 1978) to digest the particle. Neutrophil granules are also capable of releasing their

contents such as acid hydrolase, an enzyme that digests and degrades the engulfed pathogen.

Neutrophils are the primary cells responsible for the immune response. Macrophages are abundant in every tissue of the body and result from the differentiation of monocytes. They are involved in apoptosis. Along with neutrophils, macrophages also play a role in phagocytosis where they are able to secrete toxic peroxide chemicals to kill the foreign invader within the phagosome. Aside from this, macrophages also play a role in activating immune effector molecules where its role is highlighted in linking adaptive immunity. The main function of DCs is to present antigen material on their surface to other cells of the immune system, hence their term 'Antigen presenting cells' (APC). Immature DCs (iDCs) have high antigen capturing activity and low T cell activation properties. In this immature state, they function to sample the body's environment and recognize bacteria and viruses through PRRs expressed on their surface known as Toll like receptors (TLRs) (Janeway and Medzhitov, 2002). These iDCs serve to phagocytose the pathogen and degrade the antigen into small pieces and present them on their cell surface using Major Histocompatibility Complex II (MHC). This process enables the DC to undergo maturation and the mature DC migrates to the lymph node (Bell et al., 1999) where it presents the antigen to naïve T cells of the adaptive immune system and activates them. In this regard they are very important in bridging innate and adaptive immunity.

1.3 Cellular components of the Adaptive System



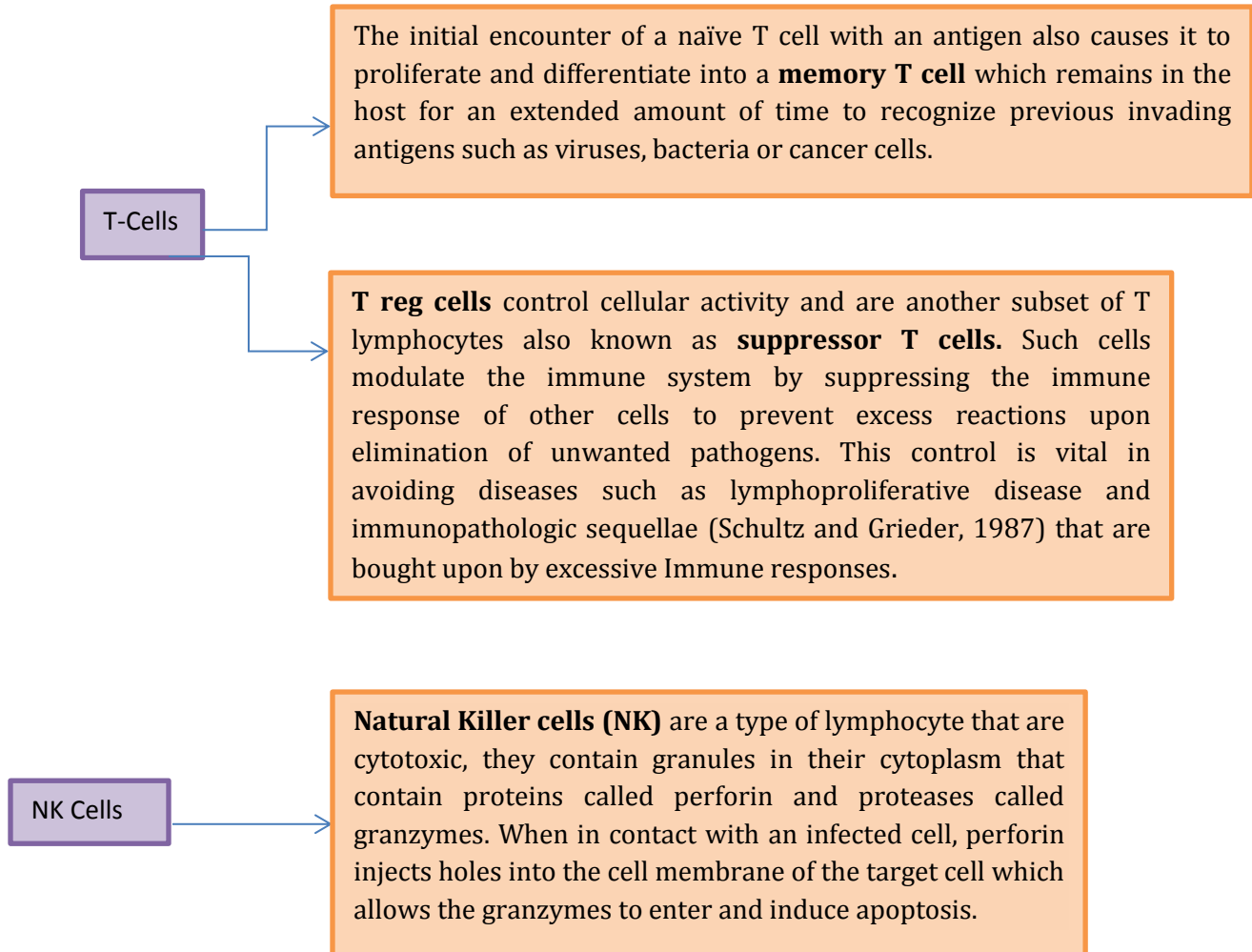


Figure 1.1: Cellular components of the adaptive system: The two main types of lymphocytes are B cells and T Cells. B cells are involved in humoral immunity and have antibodies (immunoglobulin) attached on their surface which binds to its own specific antigen to mark the invading pathogen for removal. This in turn stimulates the B cell to differentiate into a plasma cell or a memory cell. MHC molecules are found on the surface of APCs. Infected cells are able to use MHC II to display antigenic peptides on their surface to attract a compatible T cell via its T Cell Receptor (TCR). However co-stimulatory molecules on both the T-Cell and APC are required to initiate further immune responses. Upon interaction between these co-stimulatory molecules, the T-Cell can take several routes such as T- Cell activation, tolerance or T-Cell death (Abbas et al., 2004).

The primary lymphoid organs involved in the immune system include the bone marrow and the thymus. The bone marrow is critical to the immune system as it is this organ where all cells including red blood cells, white blood cells and lymphocytes are derived. Immature thymocytes migrate from the bone marrow into the thymus. The thymus is essential in providing an environment for the maturation of T- lymphocytes with the help of the hormones thymopoietin and thymosin. During this process T cells are able to differentiate between the body's cells and foreign antigens and are released into the blood stream. Secondary lymphoid organs include the spleen and lymph nodes. Both B and T lymphocytes are found in the lymph nodes with T cells residing in the paracortical area and B cells in the primary and secondary lymphoid follicles. T cells and B cells are also contained in the spleen.

1.4 Components linking Innate and Adaptive immunity

1.4.1 Cytokines and Toll Like Receptors

Essentially the innate immune system works to recognize microbes known as PAMPS (Janeway et al., 1989) via PRR germ line receptors. PRRs are abundant in several groups with their own specific role such as opsonisation, activating the complement cascade or phagocytosis. TLR are a subset of PRRs expressed on macrophages and DCs and are critical to innate immunity as they allow host cells to recognize PAMPS and are able to breach immune responses through cytokine activation (Janeway and Medzhitov, 2002). Humans express 13 TLR with each TLR able to recognize its own set of PAMPs. For example, TLR4 recognises lipopolysaccharide (LPS) (Poltorak et al., 1998) a ligand unique to gram negative bacteria, whereas TLR2 recognises peptidoglycan (Takeuchi et al., 1999). TLR1 and TLR6 form heteromeric complexes with TLR2 which enhances the amount of TLR2 signaling that occurs due to the increased types of PAMPs the TLR can attract (Beutler, 2004a).

TLR are currently widely known for being the primary sensors to pathogens to activate innate host defenses, their recruitment and activation of phagocytes can directly kill microbes (Takeda et al., 2003). In light of this, an important role for TLR is to bridge innate

and adaptive immunity through inducing DC maturation to prime naïve T cells. There is accumulating evidence that innate immune recognition by TLR shapes activation of the adaptive immune response in this manner. As described previously, a naïve T cell is able to differentiate once activated through its TCR. However a fully mature DC is required to bind to a TLR ligand to achieve upregulation of MHC and co-stimulatory molecules (Banchereau and Steinman, 1998) which will in turn allow the APC to bind and activate naïve T cells. Literature to support this has come from experiments performed involving MYD88, an essential signaling protein used by TLRs. Mycobacterial extracts containing TLR ligands were injected into mice (Schnare et al., 2001) with results demonstrating elevated levels of T cell activation. However mycobacterial extracts immunized into MYD88 deficient mice failed to induce DC maturation, thereby inhibiting T-Cell activation. This experiment highlights that TLR and PAMP engagement is essential in priming adaptive immunity to bring about T-cell differentiation and activation.

Experiments performed with DCs and TLRs have revealed that T reg cells also control activation of pathogen specific T cells. Binding of TLR4 to LPS allow DCs to mature even without the presence of MYD88 (Kaisho et al., 2001). However, this upregulates MHC and co-stimulatory molecules, but no induction of cytokines. Interestingly in spite of activating this co-stimulatory pathway, these molecules alone have been shown to be incapable of activating T cell priming. The secretion of cytokine IL-6 released as a result of TLR binding to microbial products is essentially required to block T-cell suppression, thereby allowing activation of T-cell specific adaptive responses (Pasare and Medzhitov, 2003). This is advantageous as sustained function of T reg cells prevents self-reactive T cells to activate during an immune response to infection (Abbas et al 2006).

1.4.2 Cellular Components

The cellular components of the innate immune system (Figure 1.2) include NK cells, macrophages, neutrophils and DCs (Beutler, 2004b). Macrophages are derived from monocytes and are well distributed throughout the body's major organs such as the heart, brain and lungs, thereby ensuring they are readily available to counter invading organisms. Although key in engulfing and killing microbes, macrophages are also important in

releasing cytokines to recruit polymorphonuclear phagocytes such as neutrophils. Macrophages, along with mature DCs, present antigens to naive T cells which initiate the adaptive system. Although the innate immune system was evolved much earlier, the activation of the adaptive immune system is dependent upon the myeloid cellular innate components. For instance, the abolishment of neutrophils which are key players in combating infection would result in an immunodeficiency state. Moreover without the antigen presenting function of DCs and innate immunity cells triggered cytokine production, adaptive immune responses would be unsuccessful due to pro inflammatory cytokines also involved in recruiting and activating T-Cells (Lacy and Stow, 2011)

B cells of the adaptive system are also activated by the innate system. B cells that produce autoantibodies known as Rheumatoid factor (RF) are activated by chromatin ICs (Leadbetter et al., 2002) which bind to B cell receptors (BCRs) on the surface of B cells and are delivered to TLR9. TLR9 recognizes bacterial genomes and DNA viruses, therefore interaction of these chromatin complexes to TLR9 initiates TLR9 signaling which activates B cells and allows them to differentiate and secrete specific antibodies to chromatin antigens. This can lead to pathological consequences and autoimmunity due to B cells activating non-specifically and generating self-reactive antibodies which are implicated in the pathogenesis of auto immune diseases. This establishes an evident link between the innate and adaptive immune system in the development of autoimmune diseases.

Cytokines are released upon complement activation, a key pathway of innate immunity, or as just mentioned, by TLRs (McGettrick and O'Neill, 2007). IL-12 (Interleukin 12) is a cytokine produced by APCs and links adaptive immunity by playing a role in the differentiation of T-cells into Th cells, as well as enhancing cytotoxic activity of CD8+ T cells (Iwasaki and Medzhitov, 2010). TNF is another cytokine produced by macrophages and is involved in inflammation, (Hernandezpando and Rook, 1994). It is able to induce fever and apoptotic cell death through production of IL-1 and IL-6. Another important group of cytokines are type I interferons, this family is comprised of 13 proteins that contribute to the innate immune response against viral infection. Release of these pro-inflammatory cytokines is able to mount adaptive immune responses through the recruitment of T-Cells.

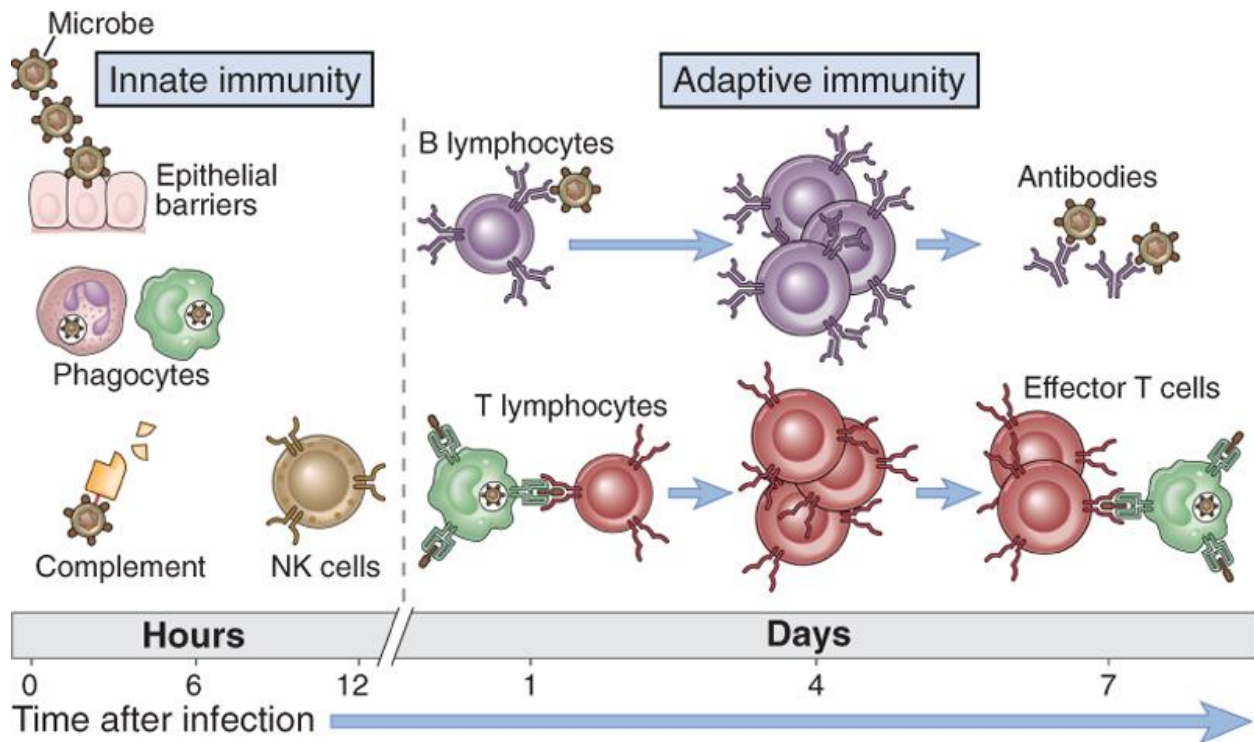


Figure 1.2: Cells and components of Innate and Adaptive immunity: The innate immune system consists of plasma proteins and cells circulating the blood and ready to fight microbes at the site of infection such as epithelial barriers, leucocytes, DCs, and NK cells. The adaptive system gets into action when pathogens evade initial innate immune responses. Components of the adaptive immune system include B-Cells that produce antibodies known as humoral immunity and T-lymphocytes ($CD4^+$ and $CD8^+$) that exert their function through cytotoxicity known as the cell mediated response (Abbas et al, 2006)

1.5 Key Innate Immune System Is The Complement System

The complement system (figure 1.3) is a key part of the innate immune system which works to remove antigen/antibody complexes known as ICs. This process is initiated by C1q, the first component of the classical pathway which latches onto the Fc portion of the antibody and triggers the activation of a range of plasma proteins, eventually leading to Membrane Attack Complex (MAC) complex which destroys the cell.

The complement pathway is made up of over 40 plasma proteins and receptors which serve as the major primary defence in innate immunity. Most of these proteins are

proteases and react with one another by proteolytic cleavage to activate themselves into their enzymatic form. Therefore, the complement system is activated via a triggered enzyme cascade. They then work to opsonize pathogens and produce inflammatory responses in response to fight infections. Eventually this amplified complement response leads to a series of events on the surface of the pathogen which works to kill the pathogen and eliminate the infection.

The complement system can be activated by both the innate and adaptive system which branch further into activating three main complement pathways (figure 1.3), the classical, alternative and mannan binding lectin (MBL) pathway (Janeway et al 2001). The innate immune activation involves the binding of mannan binding lectin, a protein that binds to mannose containing carbohydrates on the surface of bacteria. The classical pathway activation takes place when antibodies IgG or IgM bind to the antigen which exposes the Fc region of the antibody enabling the first component of the classical pathway C1q to bind to this Fc portion (Duncan et al 1988). Finally the alternative pathway is activated spontaneously when a complement protein binds to the surface of the pathogen. Although each pathway is triggered by a different mechanism, they all converge at the C3 convertase (Gigli et al., 1979).

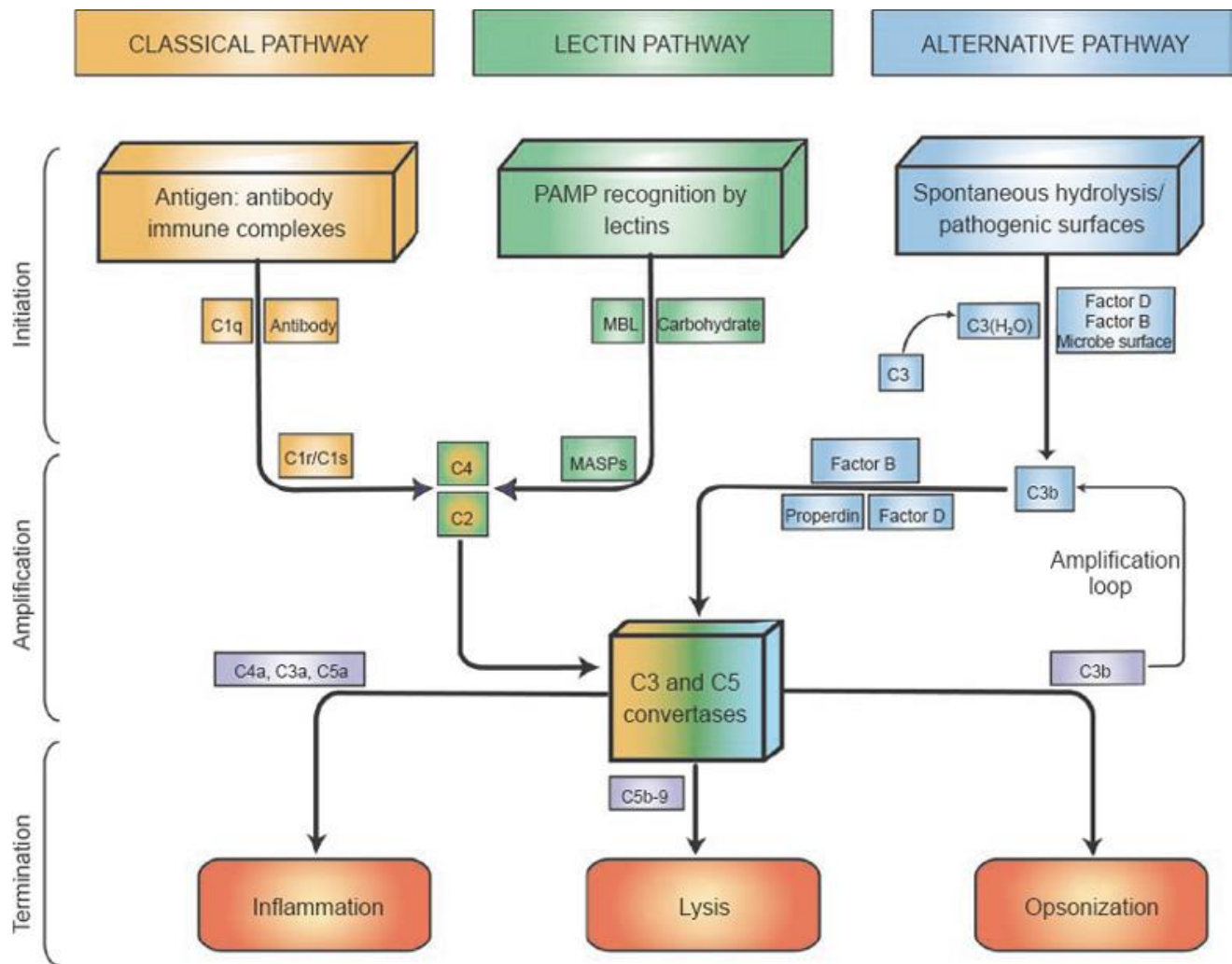


Figure 1.3: The Complement pathway: The complement pathway is activated through three pathways, the classical, Lectin and alternative. Activation of the classical pathway occurs when C1q attaches onto an antigen-antibody complex which activates C1r and C1s leading to the cleavage of C4 and C2. Lectin pathway activation occurs when MBL binds to a carbohydrate on the surface of pathogens which leads to the activation of the serine proteases MASP-1 and MASP-2, again cleaving C2 and C4. Cleavage of C2 and C4 form C4bC2a which cleaves C3 into C3b and C3a known as the C3 convertase of the classical and lectin pathway. C3b then attaches to C4bC2a to form the C5 convertase C4bC2aC3b. The alternative pathway is activated due to spontaneous hydrolysis and forms the C3 convertase C3Bb. Factor B and D lead to additional C3 cleavage forming C3bBb, the additional C3 convertase and the C5 convertase C3bBbC3b. All three pathways converge at the C3 convertase which activate the anaphylatoxins C4a, C3a, C5a and the MAC complex. Anaphylatoxins are potent inflammatory molecules resulting from cleavage of C4, C3 and C5. The MAC complex consists of the complement components C5b to C9 which form an assembly around the surface of pathogens to induce lysis (Dunkelberger et al, 2010)

The C3 convertase (figure 1.4) is the most crucial stage of the complement pathway where all three pathways converge. The protease C3 convertase activates C3 which gets cleaved into C3a and C3b. C3b binds to the surface of the pathogen and is a powerful opsonin as it mediates binding of the pathogen to phagocytic cells to target it for destruction. Initiation of the classical complement cascade begins with the binding of antibody-antigen complexes. Upon binding to the antigen, the Fc portion of the antibody is exposed allowing it to bind to the macromolecule C1 (Reid and Porter, 1976) via its globular domain. The C1 complex is 790kDa and consists of 3 subunit, C1q, C1r and C1s (Arlaud et al., 2001; Cooper, 1985) This activates the two serine proteases C1r and C1s which along with C1q make up the C1 macromolecule complex. Activation of C1r and C1s leads to the cleavage of C4 and C2 forming C4b2a, the C3 convertase. Activation of the alternative pathway arises when C3 slowly hydrolyses in solution which forms C3 (H₂O). This forms a complex with Factor B and is activated by Factor D to form the C3 convertase C3Bb. C3b binds randomly and covalently to the surface of pathogens and is stabilised by Properdin. The next stage involves the generation of the C5 convertase which cleaves C5 to produce C5a and C5b. C5b binds to C6, C7, C8 and C9 to create the C5b-9 complex known as MAC. This complex punctures pores into the surface of the pathogen and inserts into the lipid bilayer to initiate cell lysis. C3a, C4a and C5a are chemotactic factors involved in inflammation known as anaphylatoxins. These potent inflammatory molecules bring upon their effects through binding to the receptors C3aR and C5aR on monocytes/macrophages and polymorphonuclear cells to recruit the cells to the site of inflammation. C5a is the most potent anaphylatoxin and is involved in a wide range of activities including the promotion of superoxide radical production from eosinophils and the release of hydrolic enzymes from neutrophils. C3a is involved in mast cell chemotaxis (Legler et al., 1996), eosinophils (Daffern et al., 1995) and contraction of smooth muscle cells. C3aR is also known to be expressed on activated CD4⁺ T-Cells (Wefel 2000) and B-Cells. Therefore this C3a-C3aR interaction could also be involved in activating adaptive immune responses through the activation of T-Cells (Werfel et al., 2000). The deposition of C3b on complement activating fragments leads to its cleavage to produce iC3b and C3dg. These proteins are recognized by various receptors including on a range of cell types leading to the binding of the

complement particles onto the cell. The complement receptor CR1 recognises C3b and C4b. CR2 binds to C3dg/C3d and CR3 and CR4 bind to iC3b and CR1g.

The lectin pathway is activated when the protein MBL binds to Mannose residues which are exposed on various pathogens. Upon the binding of MBL to a pathogen, two serine proteases known as MASP-1 and MASP-2 are activated. Although MASP-1 was initially known to cleave C2 and C4 (Matsushita and Fujita, 1992), its role had been question and it has recently emerged that MASP-1 cannot initiate the lectin pathway (Rossi et al., 2001). Instead, MASP-1 has been identified to cleave zymogen MASP-2 to activate MASP-1 and C2 in the proconvertase C4b2, creating the C3 convertase C4b2a (Megyeri et al., 2013).

1.6 Complement protein C1q

C1q, the first recognition subcomponent of the classical complement pathway, is a 460-kDa protein composed of 18 polypeptide chains (figure 1.5) (6A, 6B and 6C) (Sellar et al., 1991; Sellar et al 1992). The A chain (233 residues), B chain (226 residues and C chain (226 residues) (figure 1.5) are composed of a N-terminal region consisting of a half-cysteine residue that is involved in the formation of disulphide bonds, a collagen-like region (CLR), (81 residues) and a C-terminal globular region, called the gC1q domain (135 residues) (Kishore and Reid 1999). There are four conserved cysteines present in each chain at position 4, 135, 154 and 171. At position 4, the cysteines present are responsible for creating the A-B and C-C dimers (figure 1.5), whereas the other three cysteine residues are involved in producing one interchain disulphide bond and one free thiol group per gC1q domain. The interchain disulphide bond is involved in creating the 6A-B dimer and the 3C-C dimer subunits (figure 1.5) (Reid and Porter 1976;Kishore and Reid, 2000). The CLR of this A-B dimer and the C-C dimer join together to give rise to a triple helical structure with the composition ABC-CBA (figure 1.5) which are held together by both covalent and non-covalent bonds. The gC1q domains contains a exposed calcium ion located near the apex (figure 1.7) which is important in target binding of its ligands such as IgM and IgG (Roumenia et al 2005)

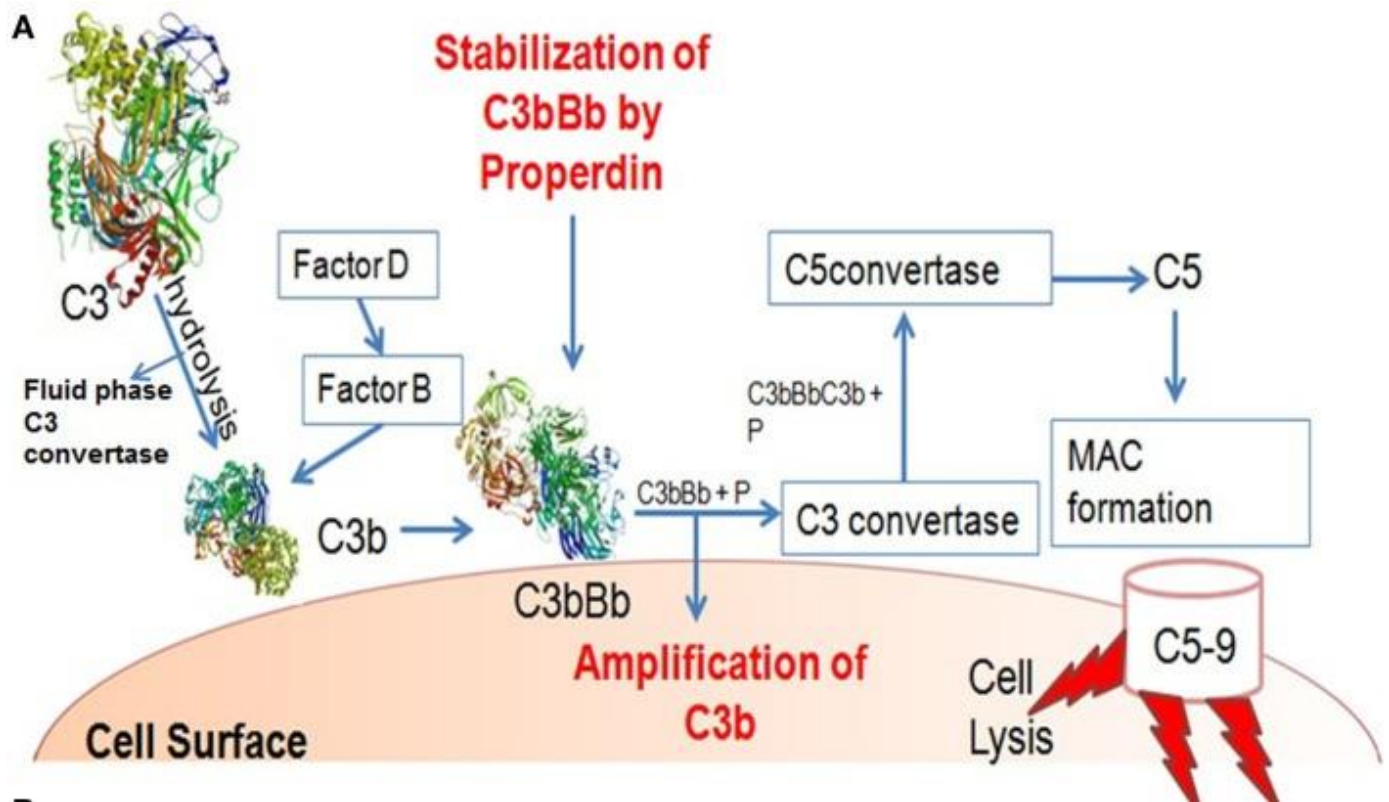


Figure 1.4: C3 convertase: Spontaneous hydrolysis of C3 causes C3b to bind to the surface of the pathogen. Properdin which cleaves more C3 to C3b amplifying the process. Surface bound C3b binds to Factor B and is then cleaved by Factor D to form C3bBb. This C3bBb is known as the C3 convertase of the alternative pathway. C3bBb is stabilized by Properdin (Kouser et al, 2013).

C1q is essentially a versatile charge pattern recognition molecule that binds to a number of ligands and triggers biological and cellular responses. This versatility of this complement protein is largely due to modular organisation of the gC1q domain (figure 1.8) (Kishore et al., 2003).

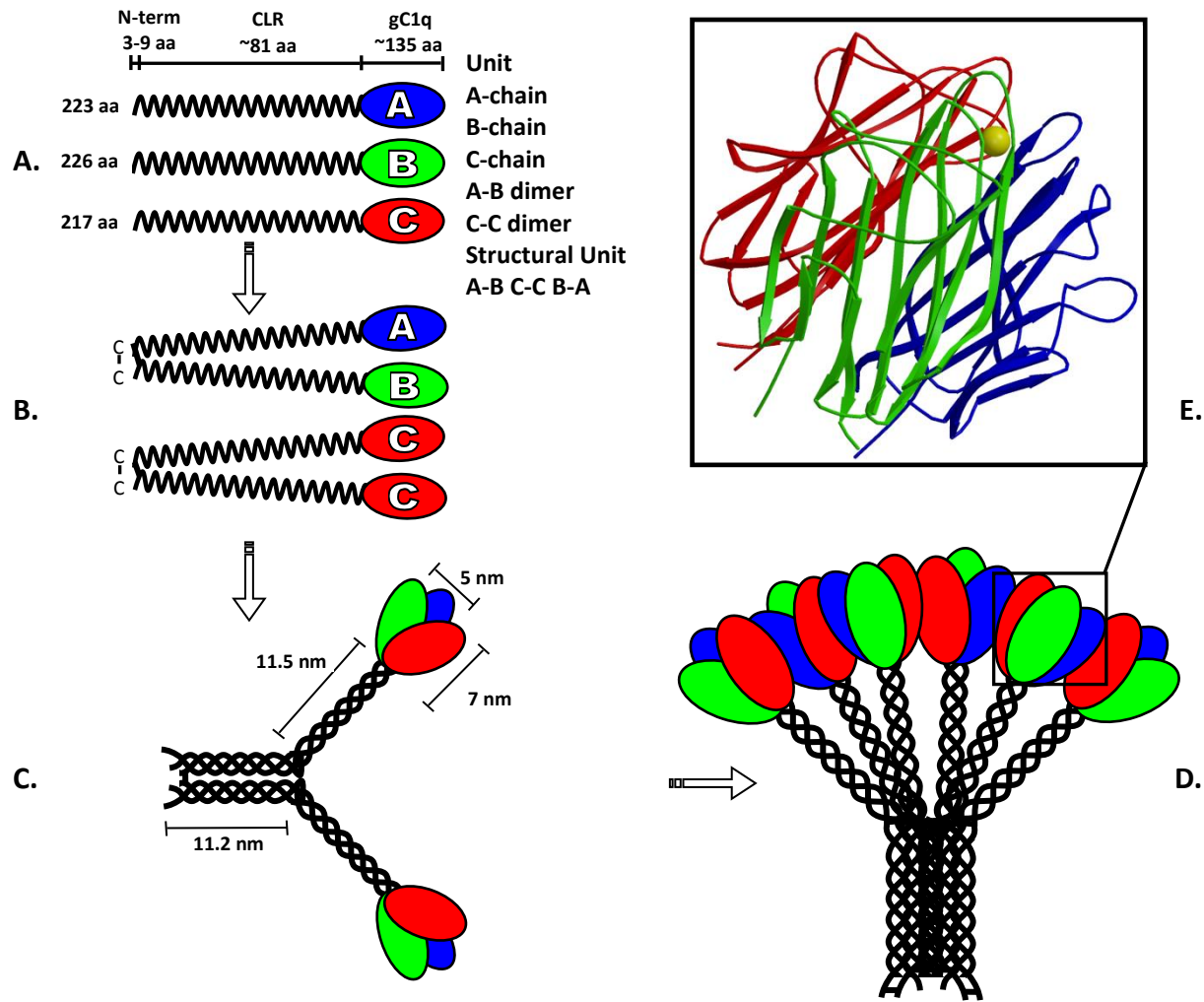


Figure 1.5: Structure of C1q: C1q is composed of 18 polypeptide chains, six C1qA chains, six C1qB chains and six C1qC chains. Each chain comprises of a N-Terminal collagen domain which resembles a stalk, and a C-Terminal globular domain. The chains are aligned as six heterotrimers which come together to create a 'bouquet of tulip' configuration. **A)** The globular head domain is made up of three different types of chains, ghA, ghB and ghC. Each chain possesses a CLR region and a C-terminal globular domain. **B)** The three heads are assembled to form disulphide linked A-B and C-C dimers. **C)** A-B-C chain. The length of each chain as well as the length of the gC1q and CLR region given in parenthesis **D)** The A-B dimer is non-covalently linked to another C chain forming A-B-C. The C chain is then linked to another C chain by disulphide bonds giving rise to A-B C-C B-A. These three doublets are linked by non-covalent bonds which gives rise to C1q's bouquet link structure (Kishore et al 1994). **E)** Ribbon representation of globular head showing ghA, ghB and ghC with Calcium ion indicated in yellow (Kishore et al., 2004).

Table 1.1: Complement activating functions of C1q

Target	C1q domain involved	Physiological response	Receptor/binding site	Reference
IgG	gC1q	The gC1q domain binds the Fc portion of IgG (figure 1.6) through ionic interactions on immune complexes (IC). Binding of this gC1q domain to IgG induces a conformational change in the CLR region which activates C1r and brings upon activation of the classical pathway. The avidity of this interaction is greatly strengthened when C1q binds to many Fc regions on aggregated IgG, as opposed to non-aggregated IgG (figure 1.6). The ghB module of gC1q is implicated in this binding with arginine and histidine residues mainly involved in assisting this complex.	The complementary binding site on IgG has been localised within the C-terminal region of the C γ 2 domain, specifically to the amino acids Glu ³¹⁸ , Lys ³²⁰ , Lys ³²²	(Kishore et al, 2003; Kojouharova et al, 2004; Duncan et al, 1988; Reid and Porter, 1976)
IgM	gC1q	Along with IgG, C1q also binds to IgM to activate complement. However, the extent of classical activation is largely dependent on the binding affinity between these two proteins. For instance, hexameric IgM binds the strongest to C1q (figure 1.6), hence activates complement to a greater degree, whereas monomeric IgM fails to bind C1q completely resulting in no activation.	Cy3 domain involving His, Asp/Glu and Pro residues at 430–434	Gadjeva et al, 2008
Endothelial cells	gC1q	C1q has also been implicated in the pathogenesis of cardiovascular diseases due to its contribution to inflammation via complement activation. C1q binds to the surface of endothelial cells via its gC1q domain leading to inflammation of the endothelium due to the buildup of ICs. The accumulation of ICs occur when patients are deficient in complement proteins leading to their inability to remove immune complexes. This build up in turn creates tissue damage, progressing to the pathogenesis of atherosclerosis	gC1qR	(Yin et al., 2007)

LDL	gC1q	<p>The pathogenesis of atherosclerosis has also been speculated to arise from C1q initiating complement activation on Low Density Lipoproteins (LDL) deposits. Enzymatically modified LDL deposits with protease and cholesterol esterase bind to C1q with high affinity and trigger C1 activation. The rationale for enzymatically modifying these lesions is to represent atherosclerotic lesions in the physiological condition of patients, as cholesterol rich particles of LDL are formed. Therefore, treatment of cholesterol esterase in an in vitro setting would expose similar cholesterol molecules that would correspond to the cholesterol rich particles of LDL, hence mimicking the physiological settings of atherosclerosis. It is this attachment of cholesterol molecules that is hypothesized to be the binding site for C1q via the gC1q domain.</p>	Not known	Biro et al., 2007)
Apoptotic cells/ blebs	gC1q	<p>Deficiency of C1q leads to the development of Systemic Lupus Erythematosus (SLE) due to the inability to remove apoptotic bodies leading to their accumulation. C1q is able to bind to apoptotic blebs and apoptotic cells through its globular head domain which effectively activates the classical pathway leading to the clearance of dying cells due to the opsonin C3b binding to the surface of the pathogen, and targeting it for destruction by phagocytes bearing the C3b receptor. Failure leads to the accumulation of apoptotic cells, marking the C1q deficiency disease, Systemic Lupus Erythematosus. A 'waste disposal' hypothesis has been described to explain the link between complement deficiency and the pathogenesis of SLE. This proposes that the deficiency of complement proteins prevents waste ICs and dying cells from being removed from the body. This in turn leads to structural modification in IC/dying cells DNA creating autoantigens that drive the production of autoantibodies seen in SLE.</p>	CRT	Nauta et al., 2002; Navratil et al., 2001; Manderson et al., 2004

		Blood platelets have been shown to bind to C1q via gC1qR and activate classical activation in response to vascular injury.		(Peerschke et al., 2006)
Platelets	gC1q		CRT and gC1qR	
CRP		Another example of how complement activation contributes to inflammation is through the protein CRP. This protein behaves as a marker of inflammation by binding to phosphatidylcholine (PS) on the surface of dying cells. This exposes its binding sites for C1q activating complement which gives rise to inflammation, leading to cardiovascular diseases.	Pore of the pentameric CRP	(Biro et al., 2007; Danesh et al., 2004)
Adiponectin	gC1q	Initiation of the classical pathway has also been demonstrated on adiponectin, the adipose specific tissue. The binding of C1q to adiponectin leading to complement activation suggests this complex could tag adiponectin target molecules such as damaged endothelium and apoptotic cells for clearance by immune responses through complement activation	Not known	(Peake et al., 2008)
PTX3		Long pentraxin 3 (PTX3) is a PRR that binds to C1q via its gC1q domain to initiate complement. However whereas immobilized PTX3 lead to C1q dependent classical pathway activation, fluid phase conditions inhibited binding of C1q with PTX due to C1q bound to IgG. This ability of PTX3 to activate and inhibit the classical pathway suggests a role for this protein via C1q in regulating the innate immune response.	PTX3 C-terminal domain	(Nauta et al., 2003)
LPS	gC1q	The globular domain of C1q recognizes LPS on gram negative bacteria with this interaction largely specified to the B chain and demonstrates classical pathway activation. In addition to this, both LPS and IgG binding sites are observed to be overlapping on the gC1q domain	LPS is mainly via the phosphate groups of lipid A	(Roumenina et al., 2008)

HAst V-1 CP	gC1q CLR	Several viral proteins also bind C1q with one of them identified as the human astrovirus coat protein (HAst V-1 CP) (Bonaparte et al., 2008). However, this interaction suppresses complement as studies have shown that classical pathway was inhibited at its first component C1 with HAst V-1 CP specifically recognizing the A chain of C1q. Since complement has great inflammatory potential, inhibiting this pathway would support the rationale behind the lack of inflammation observed in HAst V-1 gastroenteritis	Not known	(Bonaparte et al., 2008)
fibromodulin, osteoadherin chondroadherin	gC1q	Rheumatoid arthritis (RA) and Osteoarthritis also involve contribution from complement activation giving rise to chronic inflammation. This is brought upon by short leucine rich glycoproteins (SLRP) such as fibromodulin, osteoadherin and chondroadherin binding to C1q. The cartilage component fibromodulin recognizes C1q with a high affinity which could potentially account for the inflammation seen in joint disease. Osteoadherin found in the territorial matrix of articular cartilage is also known to be a very potent activator of complement; however chondroitin initiates complement to lesser complement as it binds C1q with lesser affinity	Not known	(Sjoberg et al., 2009; Sjoberg et al., 2005)
B-Amyloid	gC1q	Complement activation also follows binding of C1q to A β 1-42, the fibril peptide found in brains of patients with Alzheimer's Disease (AD). This is evident in the presence and absence of C1 inhibitor. The biological relevance of this C1 complex to activate the classical pathway supports the notion that complement activation may also be a component of neurodegenerative disease pathology by contributing to the inflammation and immune mediated damage viewed in AD.	Acidic N-terminal 1-11 region of the b-amyloid peptide	(Webster et al., 1995; Tacnet-Delorme et al., 2001)

Prion Protein

Aside from activating the classical pathway, C1q's role in targeting and scavenging altered self substances is further highlighted through its function in binding to the pathological form of the prion protein (PrP). Prp is a alpha helix rich 35 kDa glycoprotein found in neuronal tissue. Upon conversion into an abnormal isoform, prion diseases develop which represent neurodegenerative disorders. It has been speculated that complement activation occurring in prion diseases facilitates both the pathogenesis of the diseases as well as the neuronal damage. As well as this, it has been shown that the three genes for C1q are highly upregulated in the brain of mouse prion diseases. Characterization between the Prp and C1q interaction has indicated that C1q binds to the Prp through its globular head only under the condition of the Prp converted into Beta rich oligomers leading to activation of the classical pathway. More recently it has been discovered that small sized Prp oligomers of 8-15 molecules are specific to interacting with C1q and these sites initiate the highest complement activation. Moreover, C1q has also been discovered to promote Prp aggregation leading to the activation of complement pointing out a clear role of C1q in prion disease.

(Bailly et al., 2004)

(Gehlenborg et al., 2009)

(Klein et al., 2001)

(Erlich et al., 2010)

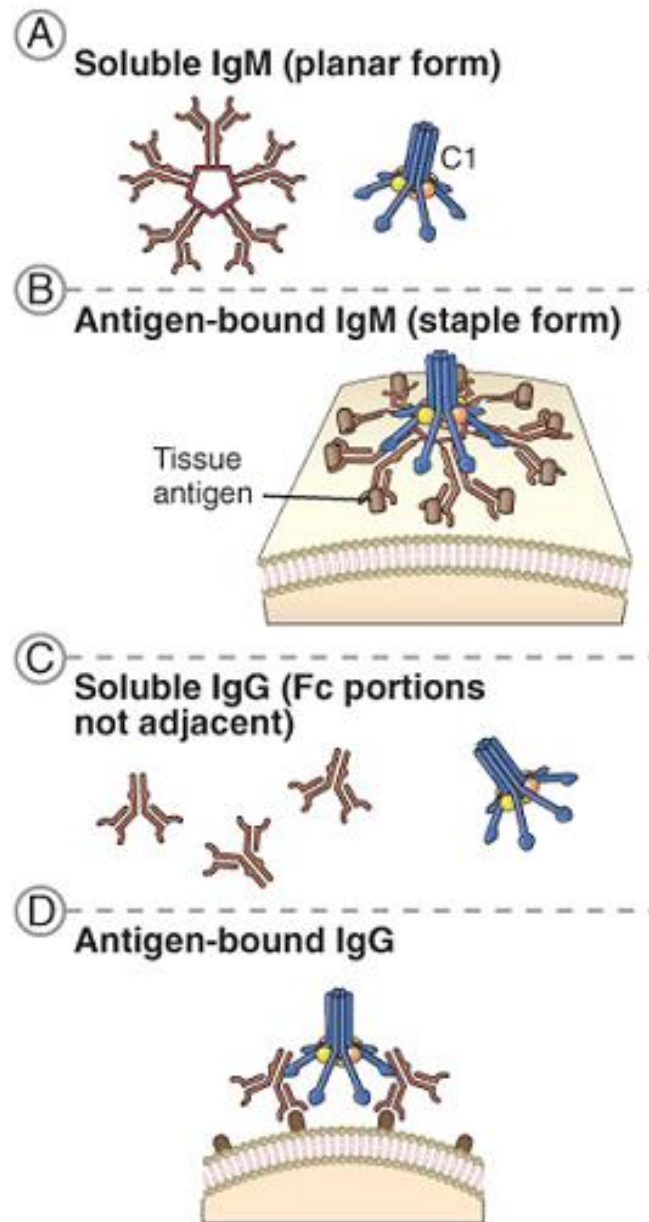


Figure 1.6: IgM and IgG antigen binding: A) Soluble hexameric IgM in its planar form. B) C1q is able to bind to hexameric IgM via its gC1q domain and activate the classical pathway, as opposed to monomeric IgM. C) Soluble aggregated IgG. D) C1q binds to aggregated IgG resulting in activation of the classical pathway.

Table 1.2: Non Complement Activating Functions of C1q

However, C1q also participates in a range of processes that are not dependent on complement activation (table 1.2) (Nayak et al., 2010).

Target	C1q doman involved	Physiological Response	Receptor/Binding Site	Reference
Phosphotidylserine	gC1q	Although apoptotic cell clearance is facilitated by the activation of complement, it is also dependent on C1q without the classical pathway. C1q has been shown to bind to PS exposed on the surface of apoptotic cells and targets them for clearance by phagocytes. C1q also possesses the ability to bind to self DNA on apoptotic cells, hence targeting them for clearance by macrophages	Not known	(Paidassi et al., 2008)
DCs	Not known	The notion that C1q is a multi-potent molecule is also reinforced due to its involvement in the maturation of DCs. Experiments have shown that immature DCs cultured on C1q activate and produce elevated levels of the cytokines IL-12 and Tumour Necrosis Factor (TNF) -alpha, as well as triggering translocation of NFkB, all components that are required for DC-maturation and T cell stimulation. Furthermore, C1q matured DCs direct a Th1 response. Immature DC start expressing CD83, CD86, MHCII and CCR7 in the presence of C1q, the upregulation of these markers of DC maturation and T cell activation suggest that immature DC that produce functionally active C1q may bind to the cell membrane once released and contribute to DC maturation. This functional role of C1q in DC biology provides an insight into how this complement protein shapes adaptive immunity responses as well as postulating the idea that the	CRT, gC1qR	(Castellano et al., 2004; Csomor et al., 2007; Castellano et al., 2004)

		absence of C1q could possibly lead to impaired functioning of DCs. Together, the idea of C1q behaving as an opsonin for apoptotic cells, and additionally regulating DC function emphasizes the concept that C1 containing ICs could also regulate immunity.		
Apoptotic Cells	gC1q, CLR	Opsonised apoptotic cells use multiple 'eat me' signals to mark themselves for removal. This hypothesis is supported by C1q recognizing Calreticulin (CRT) the candidate receptor for its CLR region on apoptotic cell surfaces which in turn attracts phagocytes to subject apoptotic cells for phagocytosis through CD91-induced macropinocytosis Therefore the prospect of the C1q globular region binding to PS and its collagen region binding to CRT elicits the idea of C1q being a multi potent protein able to collect structural features and sense multiple signals as a major player in immune tolerance.	CRT	(Paidassi et al., 2008) Ogden et al., 2001)
Heparin	CLR	C1q's non complement associated functions is also apparent in pregnancy. Heparin (Low Molecular Mass Heparin) binds to C1q via its collagen region and inhibits complement activity which entails C1q's protective role in pregnancy to prevent fetal loss	Not known	(Oberkersch et al., 2010)
		Although C1q has been linked with the neurodegenerative pathology of AD via activating complement through interacting with fibrillar amyloid peptide, evidence has also stated that C1q produces a neuroprotective role , independent of its complement functions due to no complement cascade proteins being detected in conditions that demonstrated this neuron protection by C1q. Neuronal	Not known	

<p>Microglia cells</p> <p>gC1q</p>	<p>C1q is produced after injury which suggests a beneficial role of this complement protein. In light of this statement, experiments have shown that Amyloid-beta (A- beta) induced neurotoxicity, signals through caspase and calpain, and incubation of C1q with A-beta only slightly inhibited caspase activation.</p> <p>C1q has also shown to suppress the production of proinflammatory cytokines IL-6 and TNF-alpha when treated with apoptotic neurons and microglia cells which again supports the idea of C1q acquiring a protective role. The upregulation of C1q following neuronal injury triggers a large abundance of the phagocytic cells, microglial cells in the CNS. Their function is to phagocytose the buildup of opsonised targets which in this case are amyloid peptides and apoptotic neurons that have been deposited. The mechanism employed to achieve this involves these phagocytic cells producing C1q. However, the activation of these microglia cells has been shown to be dependant on extrinsic C1q. Therefore, extrinsic C1q activates microglia cells to work similar to other phagocytes by enhancing the uptake of apoptotic neurons and suppressing inflammatory cytokine production of TNF-alpha and IL-6 in macrophages and DCs in the complete absence of activation of the</p> <p>complement cascade which enforces C1q's protective role in the CNS.</p>	<p>(Pisalyaput and Tenner, 2008: Farber et al., 2009)</p>
<p>Serum Amyloid Protein</p>	<p>In addition to this, SAP does not induce its pathological effects through caspase signaling and binding of C1q to SAP inhibited SAP induced neurotoxicity which highlights a neuroprotective role for C1q independent of these signaling pathways. This essentially implies a mechanism to be</p>	<p>(Pisalyaput and Tenner, 2008)</p>

		investigated that would reveal the route C1q employs to promote neuronal viability which would be of therapeutic beneficiary	
Neuronal Blebs	gC1q	C1q has also shown to suppress the production of proinflammatory cytokines IL-6 and TNF-alpha when treated with apoptotic neurons and microglia cells supporting the idea of C1q acquiring a protective role.	(Fraser et al., 2010)
Platelets	gC1q CLR	Platelet aggregation in response to C1q follows expression of P-Selectin and alphaII beta/beta 3 integrin. P-Selectin is involved in recruiting immune cells to the site of injury and alphaII beta 3 is vital in platelet aggregation in the disease thrombosis	CRT, gC1qR (Peerschke and Ghebrehiwet, 1997a; Peerschke et al 1993)
PTX3	gC1q	PTX3 involvement with C1q also plays a part in inhibiting the classical pathway and arbitrating the clearance of apoptotic cells. In physiological conditions, mature DC also produces these innate molecules PTX3 and C1q. This PTX3 binds to C1q in the fluid phase of DC microenvironment and inhibits complement activation on apoptotic cells as well as suppressing C1q's opsonin functions by inhibiting phagocytosis by DC.	(Baruah et al., 2006)

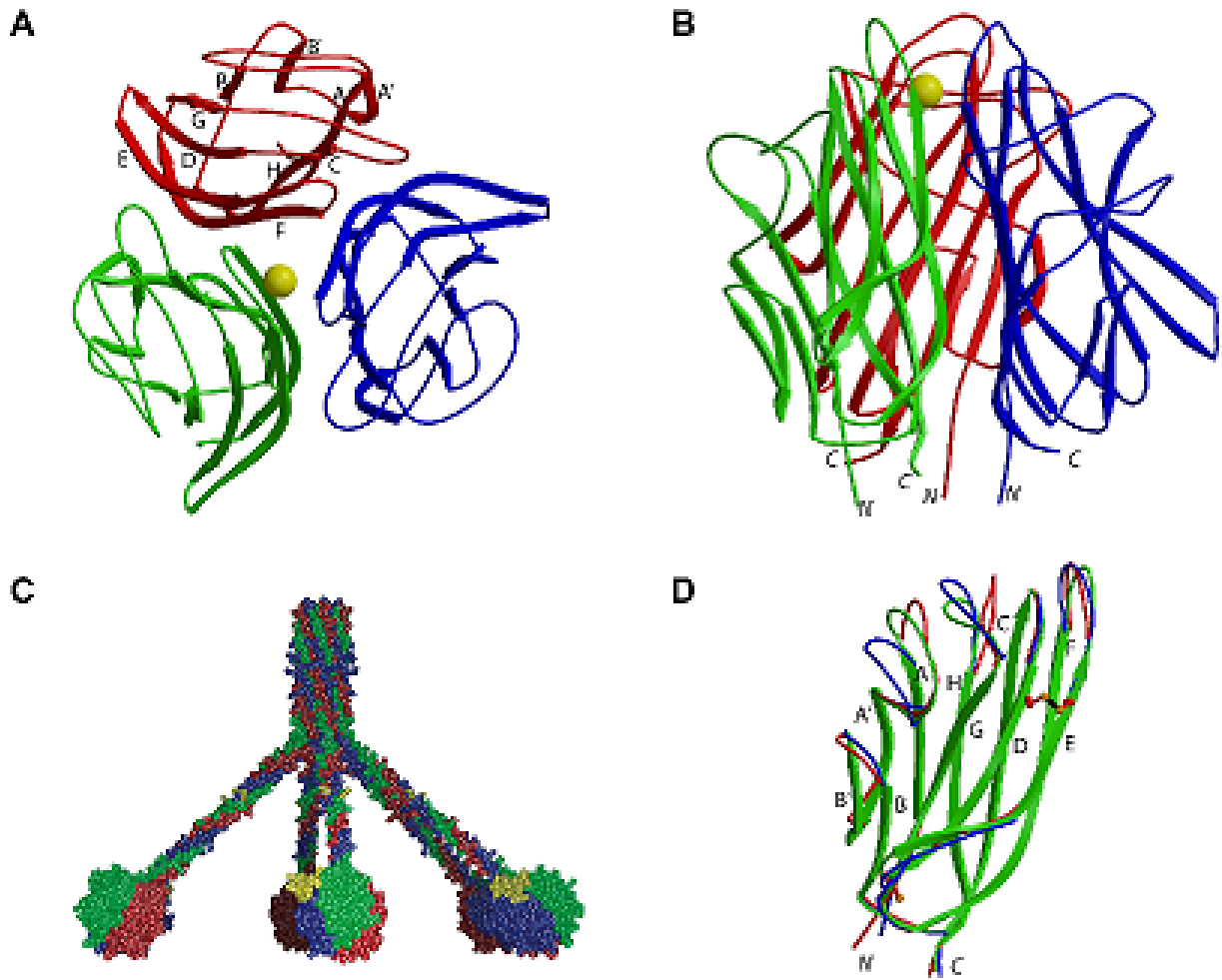


Figure 1.7: Structure of C1q: **A)** Top view of globular head domain, red: ghA, green: ghB, blue: ghC. Yellow circle represents Calcium ion. **B)** Lateral view. **C)** Lateral view of C1q showing globular head and collagen region (Kishore et al, 2004).

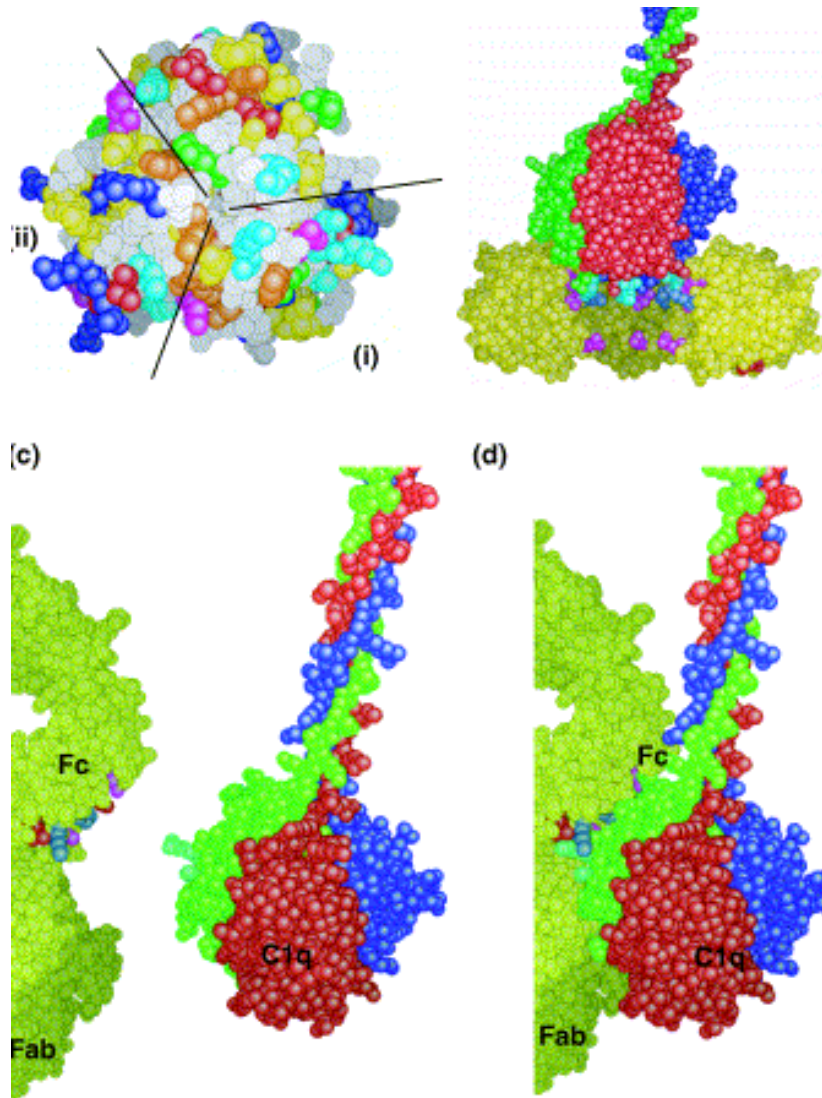


Figure 1.8: Structural basis of the versatility of C1q. The ghB module within the heterotrimeric structure of the gC1q domain is apically positioned to make contact with the Fc portion of IgG. It appears that ghA and ghC modules have subsidiary roles in stabilising the C1q-IgG interaction.

1.7 Interaction between C1q and Immune Cells:

1.7.1 Neutrophils

The nature of the interaction between C1q and a number of immune cells has been explored with results stating the enhancement of several important immune functions. These effects are brought upon through C1q binding to either its globular head receptor gC1qR or CRT which recognizes the CLR. With regards to this, the role of C1q with neutrophils to assess chemotactic properties has been studied (Leigh et al., 1998). Experiments revealed that the CLR region of C1q mediated neutrophil chemotaxis, and interestingly the chemotactic potency of C1q increased greatly in the presence of its globular head receptor gC1qR. This states that C1q functions as a potent chemoattractant via both its globular head and CLR region. In addition to this, C1q acquires the ability to trigger superoxide production in neutrophils (Goodman et al., 1995). However further studies have shown that whereas C1q's chemotactic properties function through g-protein signaling, this multi ligand protein does not utilize g protein signaling to bring upon its respiratory burst effects in neutrophils, implying different signaling mechanisms for C1q in these two functions. In addition to this neutrophils have also shown to inhibit classical pathway activation through the Defensin molecule human neutrophil peptide-1 (HNP-1) (Groeneveld et al., 2007). Defensins are antimicrobial peptides and play an essential role in antimicrobial response to microorganisms. HNP-1 is present in the granules of neutrophils and are released by stimulated neutrophils. The ability of HNP-1 and C1q to inhibit classical activation may point towards a protective role against tissue injury in inflammatory conditions.

1.7.2 Fibroblasts

C1q's chemotactic and adhesive properties have also been noted in the biological activity of fibroblasts (Bordin et al., 1990) . The adhesion capability of fibroblasts was shown to be greatly promoted when treated with C1q, with this enhanced adhesion dependant on the CLR of C1q. As C1q is known to bind to cells of the extracellular matrix, its participation in cell adhesion may highlight a mechanism of how fibroblasts adhere to molecules of the extracellular matrix. Chemotaxis of fibroblasts is also induced via the CLR (Oiki and Okada, 1988) and is dependent upon an influx of Ca²⁺ ions. As fibroblasts are known to be essentially involved in wound healing, it is

hypothesized that C1q binds to fibroblasts through a C1q receptor specified to the CLR region and triggers fibroblast chemotaxis in response to tissue injury to the site of the wound. Mast cell chemotaxis is also mediated by C1q (Ghebrehiwet et al., 1995) with the migratory response to C1q carried out in two distinct phases, a chemotactic response followed by a chemokinetic response. This could be accounted for by the presence of two separate receptors, where by the CLR binds to CRT and is then further enhanced by the binding of gC1q to gC1qR (Ghebrehiwet et al., 1995). C1q's chemokinesis properties have been further highlighted through its role in inducing migration of eosinophils (Kuna et al., 1996) through both candidate receptors gC1qR and CRT. C1q is also associated with modulating T-cell activity.

1.7.3 T-cells and DCs

C1q has also been shown to enhance IFN- γ production through T-cells as studies show that C1q produced by DC generate a Th1 response which is supported by the production of the essential defense cytokine IFN- γ (Baruah et al., 2009). This cytokine is key in driving Th1 cell mediated immune responses. In addition to this, C1q's enhanced differentiation of T-cells has been demonstrated through the mechanism of triggering CD40 ligation on DCs. As mentioned previously, C1q produced by DCs is able to bind back on DC surface. When antigen specific T-Cells recognize antigens on DC, they become activated and express CD40L. These CD40L molecules connect CD40 expressed by DCs. This leads to the upregulation of CD40 on DC which co-localise with C1q to form a signaling complex leading to enhanced T-Cell differentiation and IFN- γ production. Human DCs deficient in C1q have been shown to have an impaired response to CD40 ligation, emphasizing that C1q is required for greater T-cell differentiation.

1.7.4 B-cells

C1q stimulates the production of IgG by B-lymphocytes (Young et al., 1991) by binding to both small resting and large activated B cells. C1q also plays a role in the negative selection of autoreactive B cells (Ferry et al., 2007), C1q deficiency increases the positive selection of B1 cells and decreases the negative selection of autoreactive conventional B cells through the same intracellular antigen. This highlights a role for C1q in tolerance, in both the clearance of antigens and positive and negative selection of autoreactive B cells. Moreover, studies have shown that B cells maintain their tolerance

in the absence of C1q showing that C1q deficient cell tolerance is not a primary mechanism leading to autoimmunity, through inactivation of the classical pathway in the clearance of dying cells (Cutler et al., 2001) Another feature of this complement protein is presented through a functional role in regulating the expression of the tumor suppressor gene WOX1 in prostate cancer (Hong et al., 2009). C1q enhanced WOX1 induced apoptosis and growth suppression of prostate cancer cells through destabilizing cell adhesion. This observation has been supported through low levels of C1q promoting prostate cancer formation due to impaired activation of WOX1.

C1q also binds to the small leucine rich glycoproteins decorin, biglycan, lumican, laminin and fibronectin through its CLR. Decorin and biglycan are involved in matrix assembly and bone mineralization and bind C1q with high affinity to inhibit classical activation (Krumdieck et al., 1992; Groeneveld et al., 2005) which could have relevance in downregulating the pro inflammatory effects caused by C1q. On the other hand, Lumican, Laminin and fibronectin bind to C1q at a lower affinity and hinder complement activation (Bohnsack et al., 1985) Laminin is a large basement membrane glycoprotein, and as C1q is known to bind ICs in the basement membrane, it is hypothesized that this binding is facilitated through laminine. Therefore, within this structure, C1q could play a role in the deposition and retention of ICs.

Reflecting on these complement and non-complement functions of C1q, it is noticeable that C1q ligand interactions involving the gC1q domain usually leads to the activation of the classical pathway, whereas engagement of the CLR usually exhibits other biological functions of C1q.

1.8 Structure and Function of Human C1q

As stated earlier, the binding of C1q to multiple Fc regions on aggregated IgG as seen in ICs leads to activation of the classical pathway. In terms of IgM binding, classical activation is proportional to the binding affinity between these two proteins. In terms of mapping these immunoglobulin binding sites on the gC1q domain, mutational constructs of each ghA, ghB and ghC chain containing a single amino acid substitution have been generated which has allowed identification of key residues on the gC1q domain of C1q in ligand binding (Kojouharova et al., 2004; Ghai et al 2007). These mutants have supported an essential role for the B chain residues Arginine 114 and

Arginine 129 in facilitating C1q-IgG interaction, consistent with previous findings (Burton et al., 1980; Marques et al., 1993) Crystal structure data shows that the surface of ghB is mostly positively charged with a stretch of these important residues Arg114 and Arg129 present on the outside edge of the ghB module (Gaboriaud et al 2003). It is postulated that once IgG engages with C1q, the two molecules line up to allow the residues Asp 270 and Lys 322 of IgG to form salt bridges with Arg 129 and Glu 162 of ghB, with Arg^{B114} and Arg B161 stabilizing the complex (Ghai et al., 2007). On the other hand, the gC1q binding sites for IgM have been identified as Arg B108, Arg B109 and Tyr B175 (figure 1.9) (Kojouharova et al., 2004). Crystal structure analysis illustrates that C1q interacts with IgG at the top of the gC1q domain due to the location of these three residues. Therefore, the organisation of the gC1q domain allows recognition of IgG and IgM through different residues (figure 1.9), highlighting the structural versatility of C1q. In comparison to how the individual globular head behave when in contact with IgG and IgM, results by Kishore et al have shown the following; ghA binds IgG and IgM equally, whereas ghB binds IgG preferentially over IgM, and ghC showed better binding affinity to IgM. The difference in this functional behavior of each globular head suggest that the gC1q domain is organized in such a way that each module is structurally and functionally different (Kishore et al, 2003)

Binding of substitution mutants of ghA, ghB and ghC modules to IgG and IgM

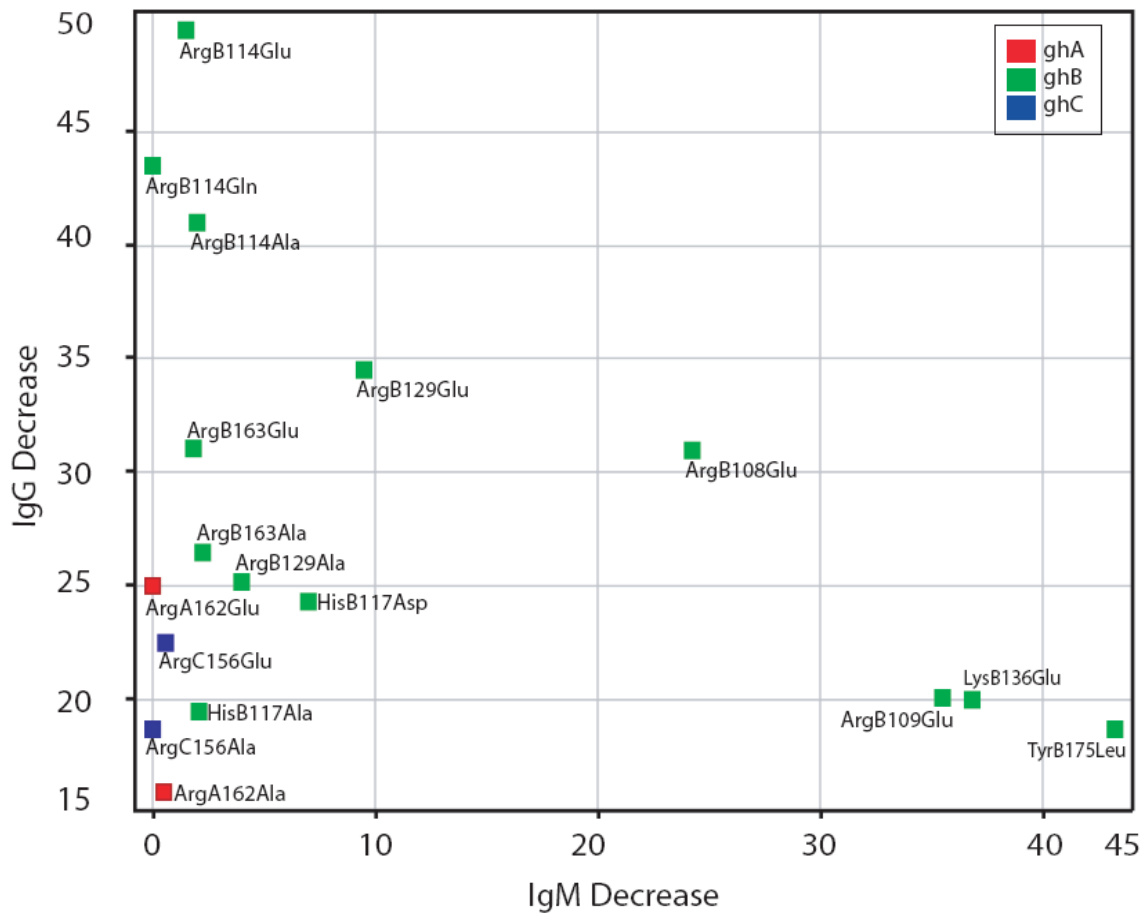


Figure 1.9: Comparison of binding decreases obtained with IgG and IgM: A scatter plot showing the % decrease in binding obtained with IgG and IgM is shown for all mutants in the study. The points on the plot are colored according to the gC1q subunit (ghA, ghB or ghC). Clearly, the ArgB108Glu and ArgB109Glu mutants (along with LysB136Glu) show highest binding decreases both with IgG and IgM, suggesting the possibility of an overlapping binding site for both on gC1q.

1.9 C1q Family

The C-terminal of C1q is also found in many other proteins, both collagenous and non-collagenous, and are recognized as 'C1q family members' (figure 1.9) (Kishore et al 2004). This C1q family is further split into two groups, a family of C1q like and cerebellin proteins, and a smaller group of EMLINS and Multimerins (figure 1.9) (Ghai et al., 2007)

Phylogenetic tree of Human C1q domain family members

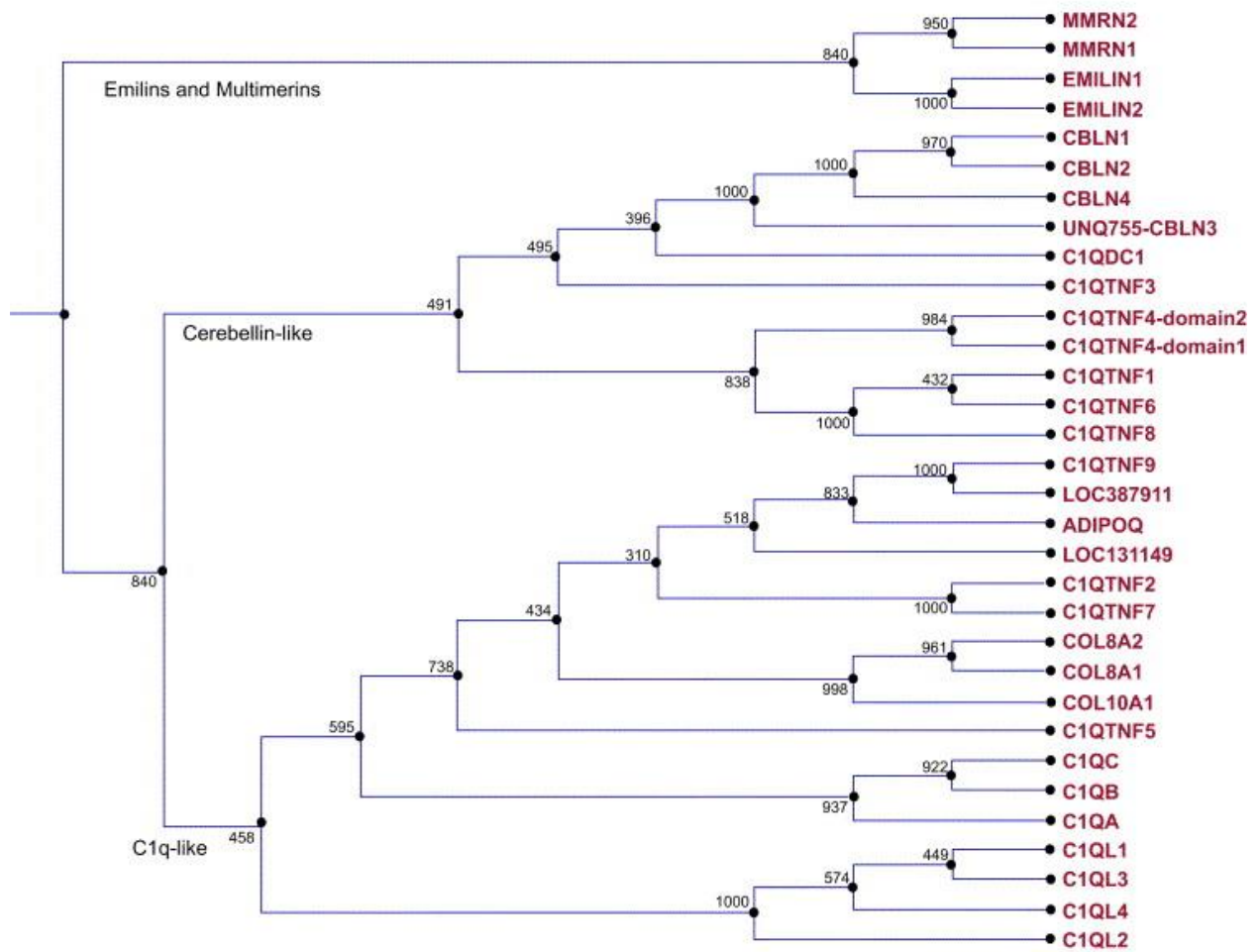


Figure 1.10: Pytogenetic tree to show C1q family members, 32 gC1q domains have been found in the human genome. C1qTNF4 is the only protein found with two gC1q domains (Ghai et al 2007)

The C1q like proteins consist of C1qA, C1qB, C1qC, COL8A1, COL8A2, COL10A1, C1QL1, C1QL2, C1QL3, C1QL4 and Adiponectin. Adiponectin is produced by adipocytes (Scherer et al., 1995) and low levels of this protein is associated with type II diabetes (Kondo et al., 2002), with Insulin greatly boosting its levels. The Cerebellin proteins are known as Cbln1, Cbln2, Cbln3 and Cbln 4 (Shimono et al., 2010) and are expressed in the CNS system. Cbln1 plays an essential role in the formation and maintenance of synapses (Matsuda and Yuzaki, 2011) whereas Cbln 2-4 are involved in the developing and maturing of brains. Out of the C1q like proteins, C1QL3 also has neuronal relevance as it is highly expressed in glial cells in areas of the brain involved in motor function (Berube et al., 1999) The C1q like collagens COL8A1 and COL8A2 are expressed in tissues undergoing active remodeling and play a role in vascular tissue development (Hopfer et

al., 2005). COL10A is localized in chondrocytes and encodes the alpha chain of type 10 collagen. Defects in the gC1q domain in COL10A1 leads to Schmid's Metaphyseal Chondrodysplasia (SMCD) which is a disorder of the skeletal structure (Dublet et al., 1999) Moving to the second subset of the C1q family members, EMLIN1 is an extracellular matrix glycoprotein expressed in elastic fibres and blood vessels, and is therefore considered to play a role in adhesion of smooth muscle cells to elastic fibres. Interestingly this function is thought to be mediated via its gC1q domain at the C-terminal (Doliana et al., 1999) EMLIN-2 is identified to be involved in the development of heart chambers (Doliana et al., 2001) and also possess both a gC1q and CLR (figure 1.11). Multimerin 1 is a large protein abundant in platelets and endothelial cell bodies, and upon exposure, binds to the cell surface of these platelets and endothelial cells to mediate platelet storage and stability (Hayward and Kelton, 1995). Multimerin 2 on the other hand is involved in vasculogenesis and angiogenesis.

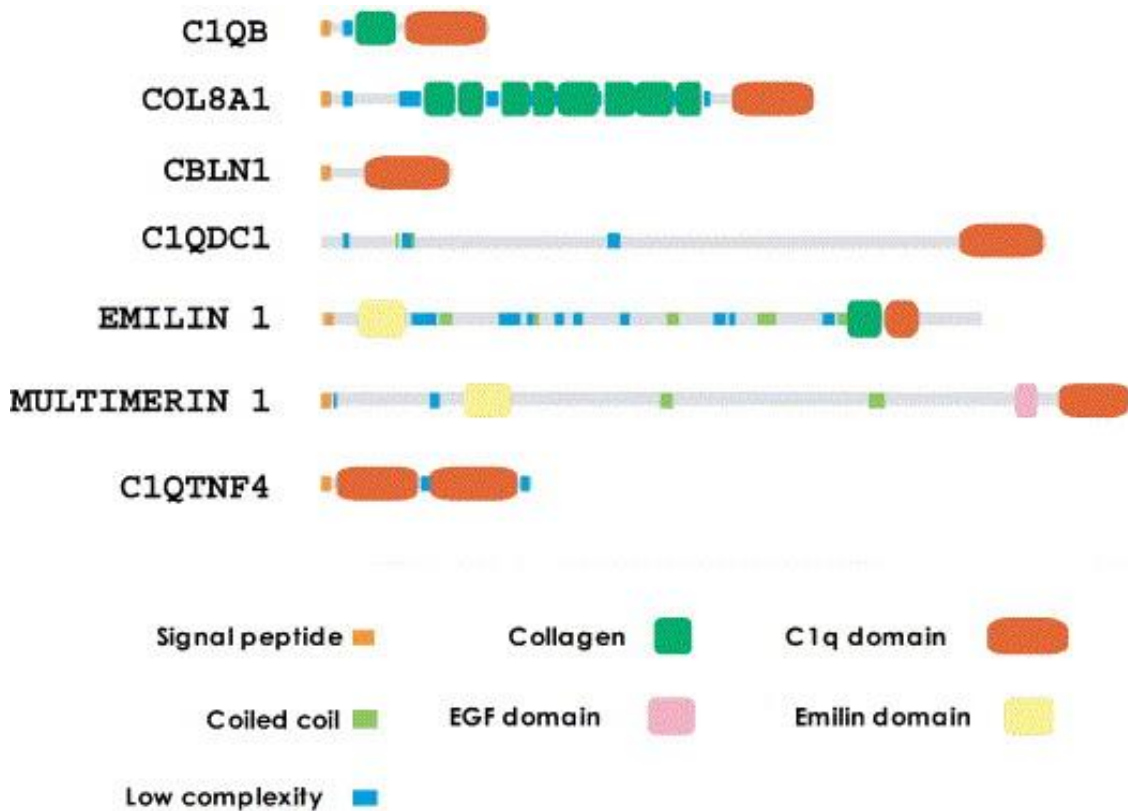


Figure 1.11: C1q like proteins representing different domains present within them in the human genome: The diagram reveals that most of the proteins have a collagenous region that appears before the gC1q domain. Each protein has a homotrimeric or heterotrimeric structure which assists in forming the trimeric structure. Multimerin 1, CBLN1, 2, 4, C1QTNF4, 5 and C1qDC1 lack collagen repeats in their domains. (Ghai et al 2007)

A new group of proteins known as C1q and TNF related proteins (C1qTNF1-9) have recently emerged to show functions similar to that of C1q and TNF members (Kishore et al 2002). C1TNF1 is highly expressed in the heart as well as the liver and skeletal muscle and plays a role in platelet aggregation (Lasser et al., 2006) C1TNF3 is expressed on fibroblasts and adipose tissue and is involved in angiogenesis and smooth muscle proliferation. Mutations in the gC1q domain of C1qTNF5 results in abnormal adhesion between the retinal pigment epithelium Bruchs membrane leading to severe vision loss.

Binding studies performed using individual ghA, ghB and ghC chains advocate that each module functions independently in binding ligands (Kishore et al., 1998). Analysis of the crystal structure shows that each gC1q domain is a spherical heterotrimer with a three-fold symmetry. Each globular head unit has a jelly roll topology consisting of two five strand anti parallel beta sheets to form a 10 stranded β sandwich (Gaboriaud et al., 2003). The interactions that contribute to holding the gC1q domain include mainly non polar interactions, followed by hydrogen bonds and main chain polar interactions. A Ca^{+} is also exposed at the apex of the gC1q domain and is implicated in assisting C1q-ligand interactions. Additional hydrophobic residues at the base, and polar and hydrophilic near the apex further contribute to holding the heterotrimeric assembly. The charged distribution of each globular head surface clearly vary from one another with ghA and ghC showing a spread of basic and acid residues on their surface and ghB appearing to be greatly positively charged (Gaborioud et al 2003).

The structural similarity between C1q family members within the gC1q domain has found C1qTNF9 and LOC387911 to show 96% sequence similarity (Ghai et al., 2007). An attractive feature of the gC1q domain show that nearly all members of the C1q family possess eight conserved residues which are crucial in maintaining the structural integrity of this globular structure. Along with C1q, four other configurations of the gC1q domain have been revealed in mouse ACRP30 (Shapiro and Scherer, 1998), human collagen X (Bogin et al., 2002) and mouse collagen (Kvansakul et al., 2003). Each of these structures reveal the same three fold symmetry, with the N and C terminal of each module positioned lateral to one another on the same side of the trimer. The collagen X structure displays a buried cluster of three Ca^{+} ions which enclose one located on the axis of the trimer. Acidic residues form a network of ionic bonds important in maintaining the high stability of the gC1q collagen X trimer. The Ca^{+} present in ACRP30

contributes to stabilizing the gC1q domain. The C1q Ca⁺ is well exposed at the apex and is crucial for ligand binding and complement activation (Roumenina et al., 2005). Interestingly, the ACRP30 structure reveals the same C-terminal structural scaffold as seen in the C-terminal homology domain of the TNF family. The beta strands between ACRP30 and TNF- α show identical lengths and positions (Shapiro and Scherer, 1998). The versatility of the gC1q domain is further highlighted through its binding to CRP (Volanakis, 1982).

1.10 Mutational Studies

CRP is an acute phase proteins that binds to PS on dying cells to activate complement via C1q. However, upon CRP binding to C1q, complement activation is brought to a halt after C3 convertase. The inability to form a C5 convertase prevents pro-inflammatory cytokines such as C5a and MAC complex to be generated (Volanakis, 2001). Therefore antigens bound by CRP are opsonised by C3b and tagged for phagocytosis by macrophages. Mutant studies have initially labeled residues Asp112 and Lys114 on CRP to be involved in the CRP-C1q interaction (Agrawal and Volanakis, 1994) CRP has five identical subunits held together by non-covalent interactions and arranged in a pentameric symmetry around a pore. Crystal structure analysis illustrates a cleft which protrudes from the centre of each subunit and extends to the central pore of the pentamer to where Asp112 is situated and indicated as the C1q binding site. CRP being pentameric in nature suggests that only one gC1q domain can bind to each CRP pentamer through one of the binding sites available out of the five subunits. Furthermore modeling of the structure of C1q and CRP has revealed a complimentary shape between the top of the gC1q domain and the pore of the pentameric ring which puts forward the idea of ghA, ghB and ghC interacting with CRP Arg112 and Tyr175 (Gaboriaud et al., 2003) LPS is another ligand that shows of the independent versatility of the gC1q domain (Roumenina et al., 2008). LPS is lipoglycan made up of a lipid and polysaccharide and is mainly found on the outer membrane of gram negative bacteria. It behaves as an endotoxin to elicit pro inflammatory responses. The ghB module of gC1q has been shown to be primarily involved in this interaction with LPS as seen with IgM. It appears that a few C1q ligands share binding sites or overlap, this is evident in IgG and CRP (Roumenina et al., 2006) and PTX3 and CRP (Nauta et al., 2003). Mutational analysis to map the specific binding residues in ghB that is complimentary to LPS

indicated that Tyr175 is essential in this interaction which clearly shows that the IgM and LPS binding sites are shared on the same gC1q domain.

1.11 Biosynthesis of C1q

C1q is synthesized by a range of cell types including macrophages (Petry et al., 1991), DCs (Vegh et al., 2003), decidual endothelial cells (Bulla et al., 2008) and microglia cells. Although C1q is produced in the liver, adherent monocytes and macrophages appear to predominantly express C1q (Loos et al., 1983). It has been found that formation of a functional C1q molecule on macrophages depends upon three mRNA species each coding for the A, B and C chains (Petry et al., 1991). Experiments have demonstrated that membrane C1q (mC1q) is synthesized in a developmental phase by monocyte derived macrophages. In addition to this it has also been explored whether the activation of these macrophages producing C1q affect the function of this protein. Data has highlighted a strong link between IFN- γ production in macrophages and the expression and function of mC1q, as IFN- γ increased the expression of C1q and amplified mC1q mediated phagocytosis. Peripheral blood monocytes have also shown expression of surface C1q (Hosszu et al., 2008) with this monocytic supply of C1q contributing to monocyte derived DC differentiation (Vegh et al., 2003).

C1q production is also evident in monocyte derived DCs. However, once DC seem to differ into mature DCs, C1q expression is abrogated. The differentiation of monocytes into activating DC mediated through the immunostimulatory cytokine IFN- α illustrates an inability of these mature DCs to synthesise C1q. This reinforces the idea of IFN-alpha playing a pathogenic role in SLE where low levels of C1q observed could be due to this cytokine (Ronnlom and Alm, 2001; Castellano et al., 2004). The production of functionally active C1q by immature DC (iDCs) provides an additional role for C1q in apoptotic cell clearance, as iDC show enhanced uptake of apoptotic cells through their C1q. Interstitial DC and Langerhan cells are subsets of DC and at precursor levels also produce low amounts of C1q. However, once differentiated into DC, interstitial cells appear to produce a considerably higher amount of C1q (Castellano et al., 2004). Recently, it has emerged that C1q is locally produced in the kidney by myloid DCs (Castellano et al., 2010). This has implications in the pathogenesis of lupus nephritis as it has been found that SLE patients with severe lupus nephritis showed C1q positive

myeloid DC infiltrating into the tubule interstitial which may contribute to local tissue damage seen in SLE via complement. Expression of C1q has also been detected in arterial wall DCs (Cao et al., 2003), which suggests a role for DC abundant in atherosclerosis lesions suggesting that the C1q they produce may play a role in trapping ICs as seen in the pathogenesis of atherosclerosis. Unlike other complement proteins, this APC cell origin of C1q by macrophages and DCs puts forward a role for C1q in regulating APC mediated adaptive immunity which is linked to the pathogenesis of several diseases.

Interestingly, decidual endothelial cells (DEC) have been shown to synthesise and express C1q (Bulla et al., 2008) unlike other endothelial cells of blood vessels. This surface bound C1q to DEC has been shown to act as a molecular bridge between the decidual endothelium and endovascular trophoblasts. In relation to this, migrating extravillous trophoblasts have also been shown to synthesise and secrete C1q (Agostinis et al., 2010) with their C1q promoting trophoblast invasion of the decidua through C1q interacting with Integrin $\alpha 4$ of the decidual extracellular matrix. Interestingly, mice studies have revealed that C1q deficiency led to increased fetal resorption, reduced fetal weight and impaired decidual vessel remodeling which suggests that defective production of local C1q can lead to pregnancy disorders. As mentioned previously, neurons and microglia in the CNS are also known to synthesise C1q. More recently it has emerged that astrocytes also induce the production of C1q in neurons through a mechanism that is yet to be identified (Stephan et al., 2012). Thus, the local synthesis of C1q can lead to regulation of the inflammatory processes.

1.12 gC1q binding receptors

1.12.1 gC1qR

gC1qR (33kDa) and CRT (previously known as cC1qR) are two well-studied and most ubiquitous C1q receptors involved in mediating a range of C1q mediated biological processes. The 33kDa protein gC1qR was first isolated from Raji cells (figure 1.12) and is found on fibroblasts, neutrophils and endothelial cells. gC1qR has been shown to bind to the globular head region of C1q (gC1q domain) (Ghebrehiwet et al., 1994). In addition to binding to C1q, gC1qR has also been shown to bind other various plasma proteins such as thrombin, prothrombin and vitronectin (Lim et al., 1996). Although

expressed on the cell surface, gC1qR interacts with β -integrin to allow transmission of signals to the cell surface (Eggleton et al., 1995). In terms of secretion, different studies have challenged various findings of how gC1qR is secreted. Peterson et al demonstrated that gC1qR is secreted in a soluble form from cytoplasmic vesicles on the plasma membrane of Raji cells and Peripheral blood lymphocytes). However, Dedio et al has shown that gC1qR is localised in the vesicular compartment of endothelial cells concluding that its primary location is intracellular with only a minority located on the surface. The underlying mechanism of gC1qR secretion is yet unknown (Peterson et al., 1997)

1.12.2 gC1qR Structure and Function

gC1qR is shown to bind to the gC1q domain of C1q via high affinity interactions (Ghebrehiwet et al., 1994). It has also been shown to interact with proteins of the intrinsic coagulation/ Bradykinin cascade such as HK (High molecular weight Kininogen), fibrinogen, thrombin and multimeric vitronectin (Joseph et al., 1999) Binding of gC1qR to C1q has been shown to bring upon a number of biological responses including procoagulant activity on platelets (Peerschke et al., 2001) and anti-proliferative responses on B and T cells. C1q is shown to bind to gC1qR expressed on platelets bringing upon initiation of complement, which leads to inflammation. Experiments have shown that platelets exposed to stress led to the largest amount of gC1qR expression and the highest

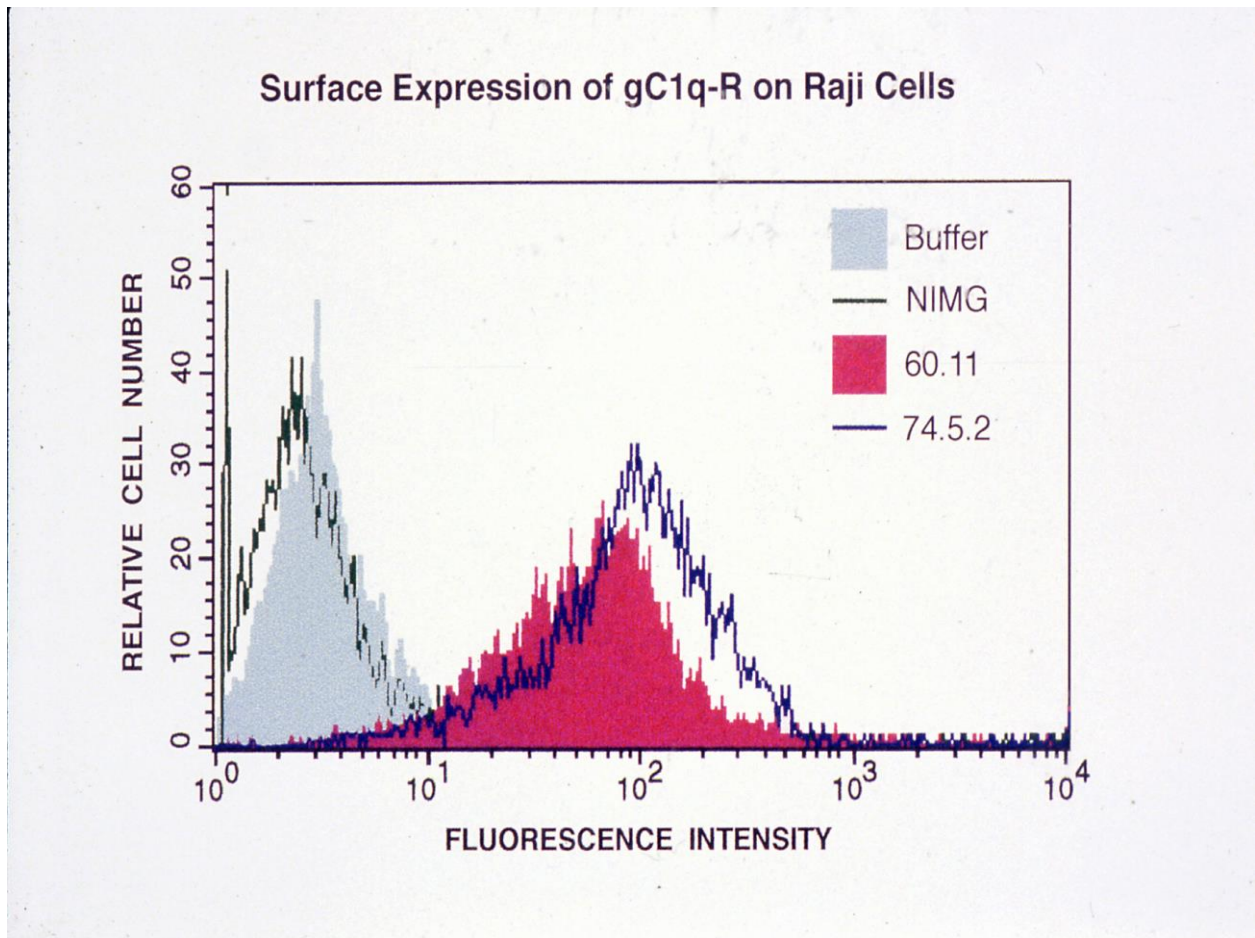


Figure 1.12: Surface expression of gC1qR on Raji cells: Antibodies 60.11 which binds to residues 74-96 and pAb 74.5.2 which interacts with residues 204-218 have been used to detect expression of gC1qR. (Ghebrehiwet et al 1994)

C4 deposition. This suggests that platelets may drive classical activation in this manner to sites of vascular injury to enhance the inflammatory response to repair wound healing.

Alongside this, C1q's non complement functions are also underlined in the activation and adhesion of platelets through binding to both CRT and gC1qR (globular head receptor) expressed on platelets. Studies have shown that platelet aggregation in response to C1q follows expression of P-Selectin and alphaIIb beta3 integrin. With P-Selectin playing an essential role in recruiting immune cells to the site of injury and alphaIIb beta3 crucial in platelet aggregation in thrombosis, C1q activating platelets and inducing procoagulant activity may contribute to the pathology associated with thrombosis. Binding of the Hepatitis C Virus (HCV) core to gC1qR expressed on the surface of T-cells weakens the activation of the lymphocyte's intracellular signaling

proteins, Lck and ZAP-70, thereby effectively suppressing T cell proliferation (Yao et al., 2004). The binding of gC1qR to HK brings upon the formation of Kalekrein, which generates Bradykinin, a peptide that increases the vascular permeability of the vessel wall leading to the infiltration of pro-inflammatory tissue (Kaplan et al., 2001).

gC1qR, being a cell surface protein brings to our interest the properties of its two faces; the membrane face which is basic and interacts with the cell surface, and its solution face which is highly acidic and recognizes ligands (figure 1.16) (Ghebrehiwet et al., 1994). The C1q binding site on gC1qR was identified on residues 76-93 (figure 1.17), whereas the HK binding side resides on residues 205-218 (figure 1.17) (Joseph et al., 1996). Since C1q binds gC1qR when it is bound to the cell surface, the C1q binding site 76-94 as expected is only exposed on the solution face (Ghebrehiwet et al., 2002).

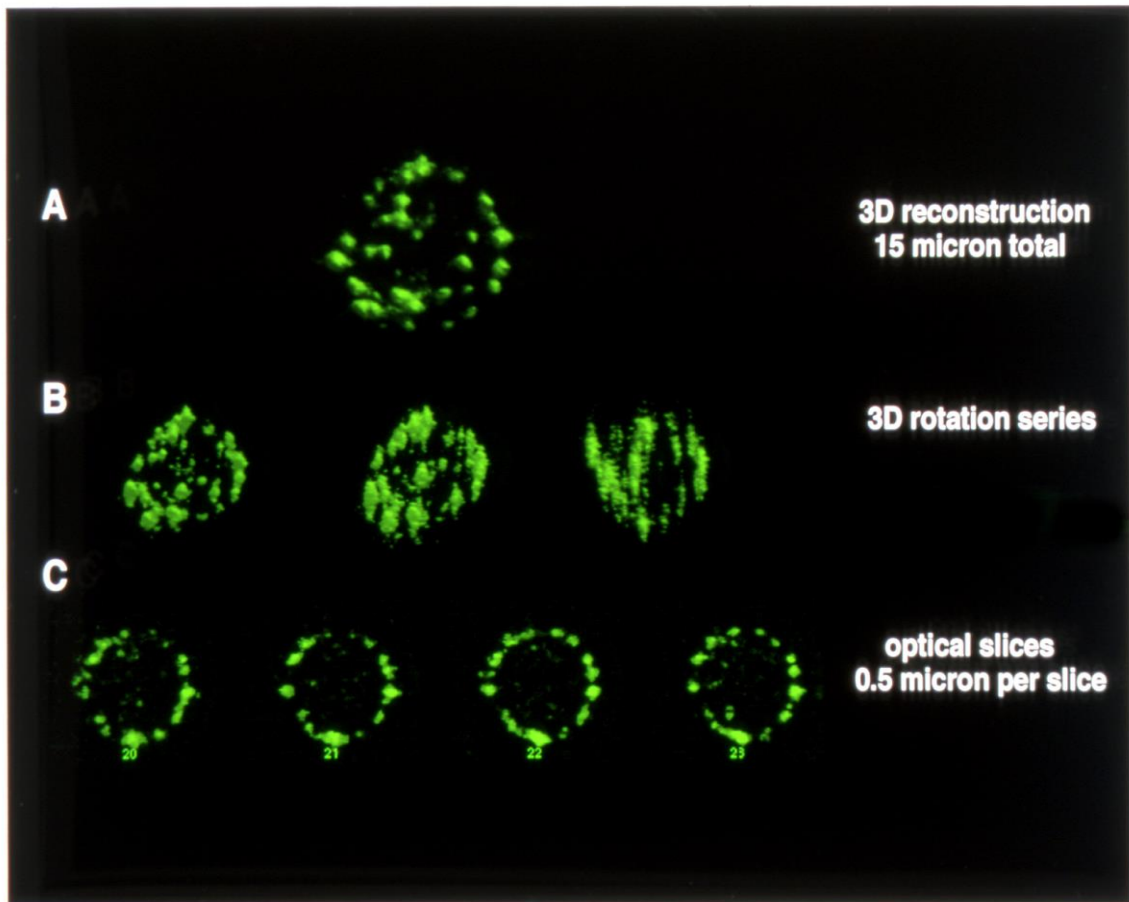


Figure 1.13: Surface expression of gC1qR in permeabilised and non-permeabilised cells. 2-D microscopic studies reveal surface expression of gC1qR as well-defined patches.

gC1qR is synthesized as a pre-pro protein of 282 residues. It was initially thought that residues 74-282 were expressed on the cell surface membrane, postulating that the pre-pro residues 1-73 are cleaved off, expressing only the 'mature' form. However, it has recently been shown that the full length protein comprising of residues 1-282 do in fact exist on the cell surface (Hosszu et al., 2010). The three - dimensional crystal structure of gC1qR resembles a trimeric 'doughnut' (figure 1.14) composed of 3 identical chains (Ghebrehiwet et al 2002). The spacefill residues between each monomer are known as the amino terminal Met, and carboxy terminal, Gln, (Ghebrehiwet et al., 2002) and between each monomer the amino and carboxy terminals are in contact with each other (figure 1.14). The crystal structure shows gC1qR folds with seven antiparallel β -strands with one N-terminal and two C-terminal α -helices (figure 1.15)

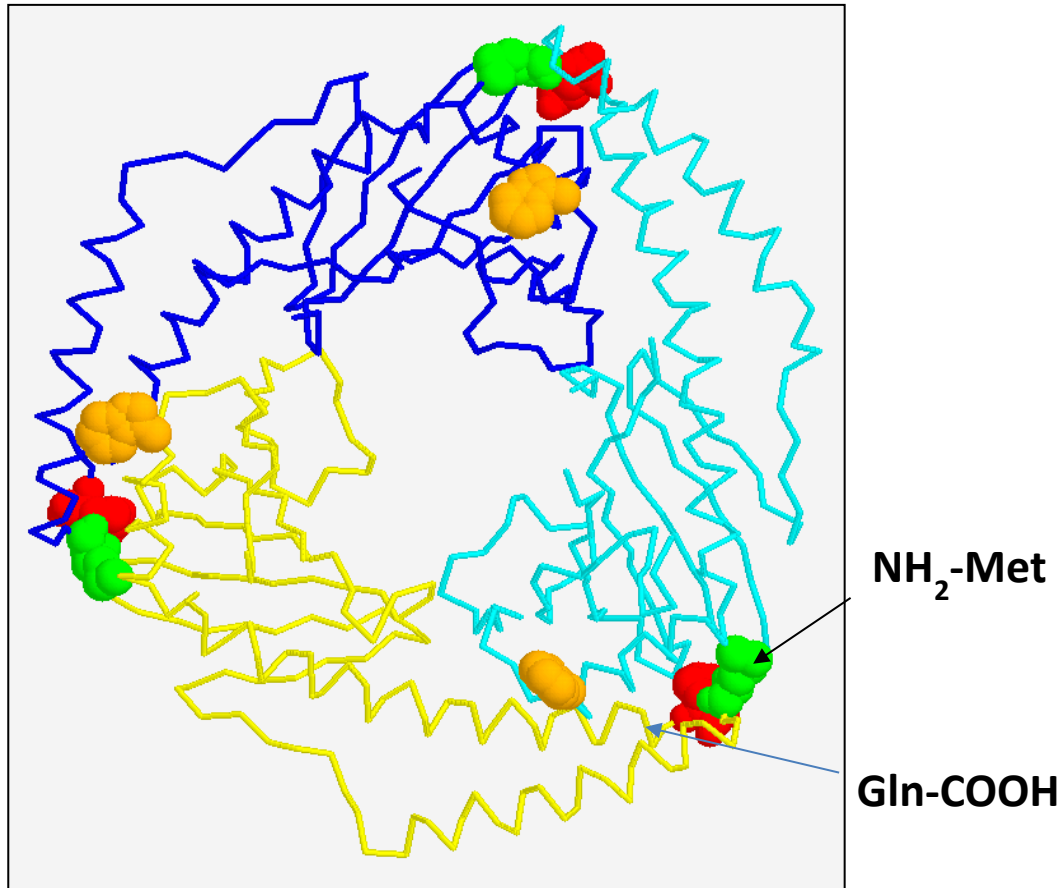


Figure 1.14: Structure of gC1qR: The configuration of gC1qR appears as a homotrimeric structure composed of three momomers. The spacefill residues are the amino terminal Met (red), the Carboxy terminal Met (green) and the carboxy terminal Gln (yellow). In each monomer the amino terminal and the carboxy terminal are in contact with each other (Ghebrehiwet et al., 2002)

As mentioned previously, an interesting feature of gC1qR is that it is devoid of a transmembrane domain, therefore, how it is anchored to the cell surface is not known. It is speculated that gC1qR transduces its signal through a docking/signaling complex via membrane spanning proteins such as β 1 integrins (Yin et al., 2007). This has been demonstrated on endothelial cells, where binding of gC1qR to its ligand (gC1q) leads to the assembly of these membrane proteins, which have direct means of expression to signaling factors within the cell. Thus, it is by this signaling mechanism that gC1qR is

able to convey its message across the membrane and bring upon biological responses such as inflammation (Feng et al., 2002)

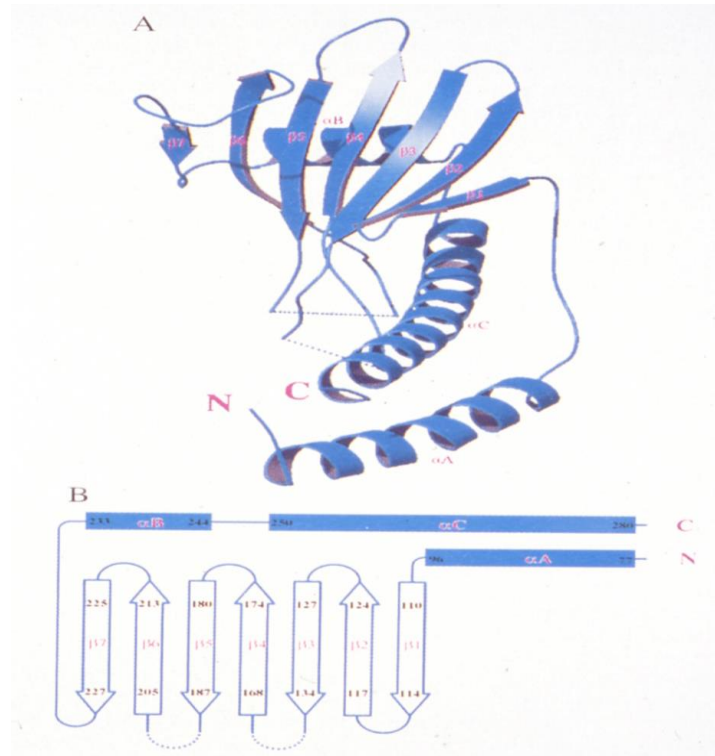


Figure 1.15: Folding topology of gC1qR monomer: Ribbon representation of gC1qR showing seven antiparallel β -strands with a C-terminal and N-terminal α -helical strands at the edge.

Although gC1qR binds to the globular head region of C1q, it is not yet identified as to which chain of the gC1q domain this interaction is specific too. To date it has been shown that C1q binding to gC1qR on platelets and endothelial cells induces complement activity independent of IgG (Peerschke et al., 2006), as well as an anti-proliferative response on B and T lymphocytes (Ghebrehiwet et al., 1990). Recently it has been found that gC1qR is associated with C1q on the surface of immature DC, along with DC-SIGN to form a trimolecular complex (Hosszu et al., 2012). As C1q is already known to modulate the differentiation of monocytic derived DCs, this provides a potential role for C1q to bind to gC1qR-DC-SIGN complex and initiate DC-SIGN mediated cellular signaling to regulate DC differentiation.

Residues 1-73 which consist of the precursor sequence have been found to hold a signal sequence MLPLLRVPRVLG at its N terminus which is targeted to the mitochondria

(Dedio et al, 1998) but could also be a signal sequence to anchor gC1qR to the cell surface. It appears that the sequence CGSLHT that forms the mature protein is identical in mitochondrial and cell surface gC1qR which hypothesizes that the signal pathway that processes intracellular gC1qR may also target gC1qR to the cell surface (Van Leuwen and O' Hare 2001)

Mature gC1qR is very acidic with a calculated p.i of 4.15. Unlike the prepro sequence which holds an abundance of Cys residues, the mature sequence holds only one Cys at position 186 so is void of intrachain disulphide bonds (Ghebrehiwet et al 2001).

The cDNA of gC1qR reveals 97% sequence homology between rat and mouse, whereas comparison between both these sequences and human shows 89% similarity ((Lynch et al., 1997). The gC1qR gene is located on chromosome 17p13.3 and is composed of 6 exons and 5 introns.

The faces of gC1q-R

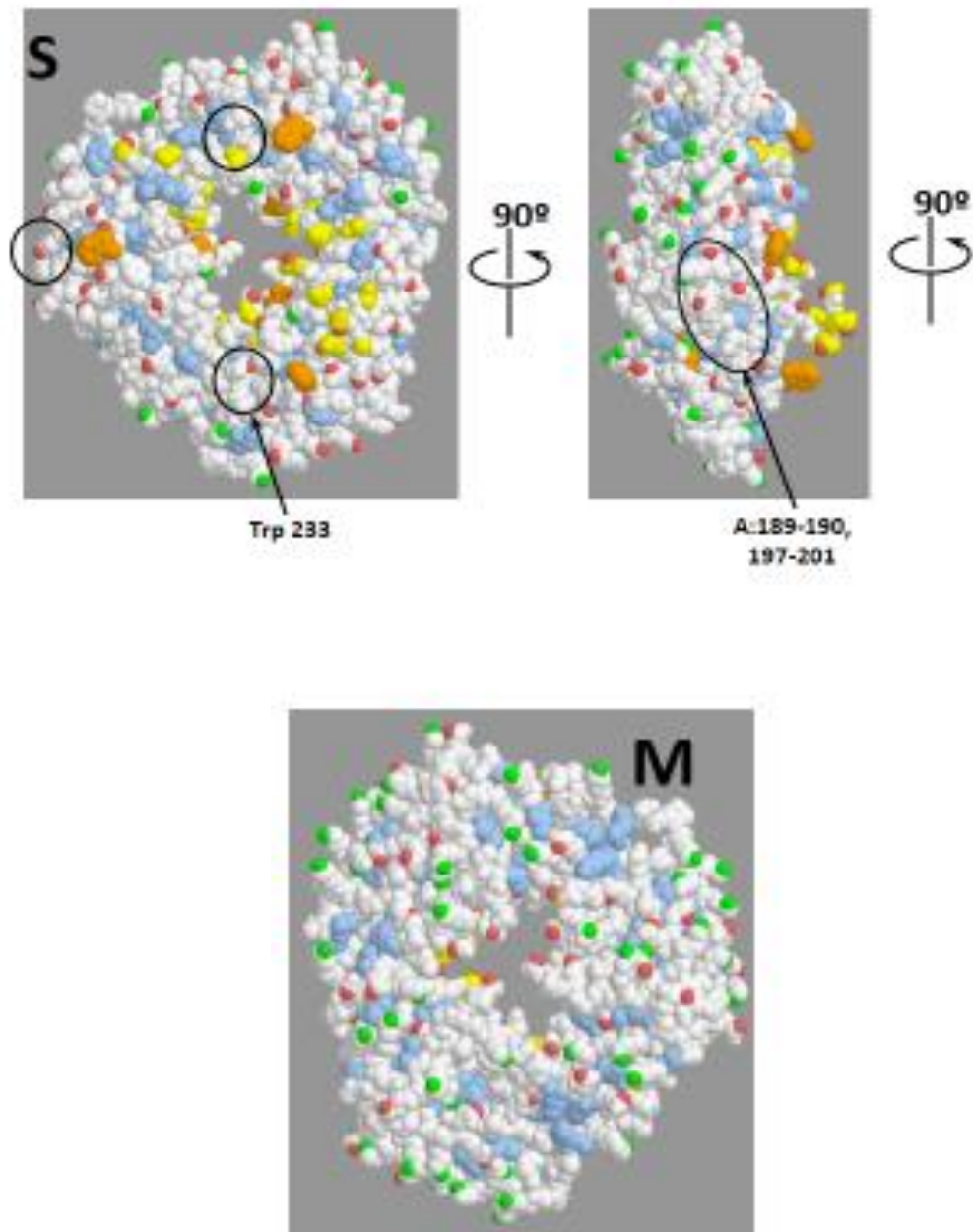


Figure 1.16: Faces of gC1qR: gC1qR consists of two faces, the solution face (S) and the membrane face (M). Residue Trp283 protrudes from the solution face. Sequences 189-190 and 197-201 are only located in the A chain. The rotation of gC1qR 90° from the S face shows residue Trp283 projecting out.

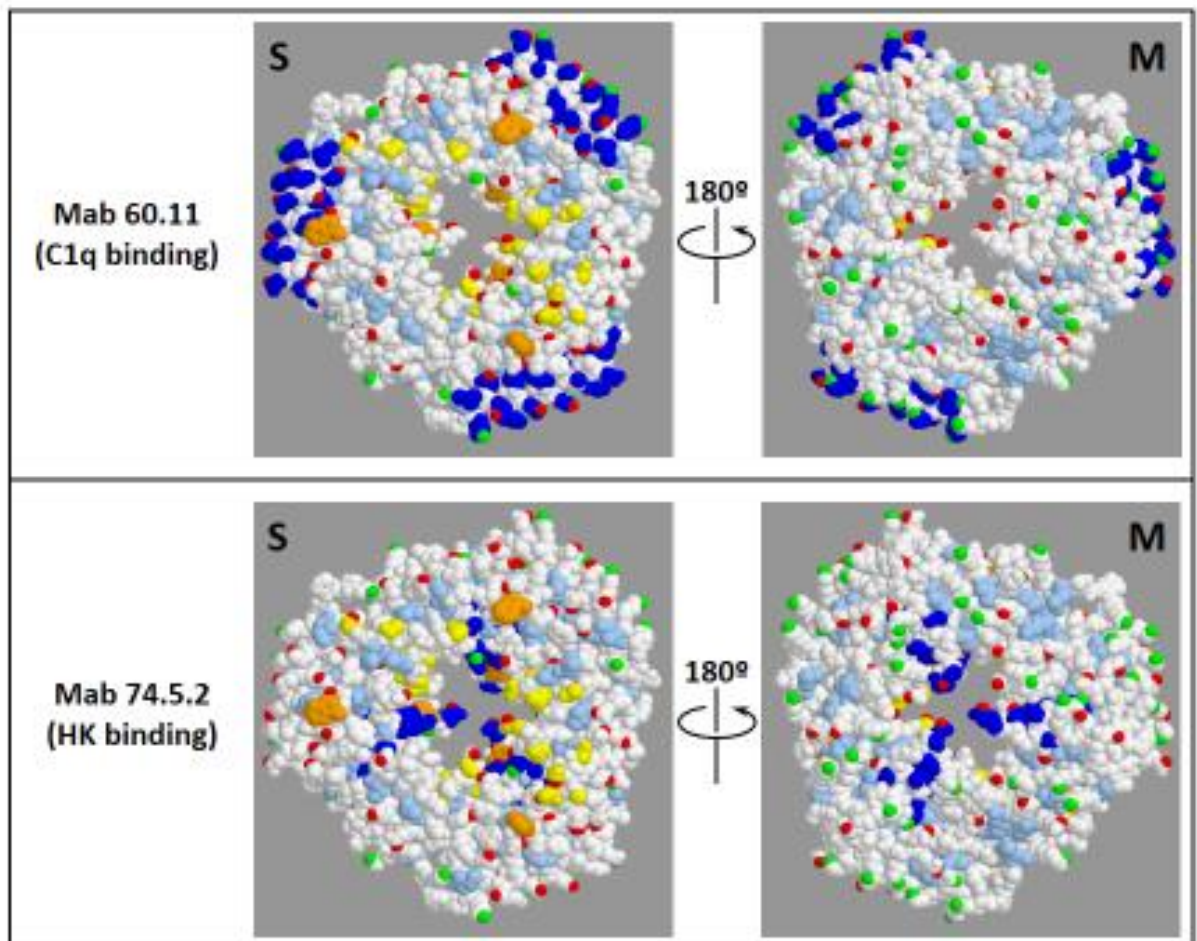


Figure 1.17: Ligand binding sites on gC1qR: Crystal structure analysis has enabled the modeling of gC1qR to identify ligand binding sites. The diagram represents the soluble (S) face and membrane face (M) when the protein is rotated 180°. The binding sites for the gC1qR ligands, C1q and HK have been identified and represented in blue according to antibody recognition. The mAb 60.11 recognizes the C1q binding site (residues 76-94), indicated in blue on S face and M face. The mAb 74.5.2 recognises the HK binding site (204-218) indicated in blue on the face and M face.

Table 1.3: gC1qR is a Pattern Recognition Receptor: Viral ligands gC1qR binds to and subsequent effector functions

Viral Ag	gC1qR (aa residues)	Encoding Exon	Function	Reference
EBNA-1	244-282	VI (234-282)	Intracellular: Regulates splicing	Wang Y et al.1997
HIV-1 Tat	244-260**	VI (234-282)	Intracellular: Regulates HIV1 splicing through interaction with p32 (gC1qR)	Yu L, et al, 95
HIV-1 Rev	196-208	V (193-233)	Intracellular: regulates viral transcription	Luo Y, et al, 1994
HIV-1 gp41	174-180	IV (159-192)	gp41 3S motif binds to CD4+ T cells-to induce NKp44L, a cellular ligand for an activating NK receptor	Fausther-Bovendo H et al.2010
Herpes simplex ORF P	ND	ND	Intracellular: regulates RNA splicing	Bruni R, et al, 1996
Core Protein V-Adenovirus	ND	ND	Intracellular-imports proteins to the nucleus and CPVA hijacks this process to deliver its genome to the nucleus	Mathews DA, et al. 1998
Core Protein HCV	188-259 (196-202)	IV-VI (159-282)	Binds to gC1qR on T cells and suppress cell proliferation and immune response	Kittlesen DJ, et al, 2000
Capsid protein-Rubella virus: N-terminal 28aa	212-282	V-VI (193-282)	Interacts with gC1qR and enhances viral infectivity	Mohan, KVK, et al. 2002
Hanta virus	ND	ND	Requires gC1qR for binding and infection	Choi Y et al 2008

gC1qR is a pattern recognition receptor due to its ability to bind to a range of viral ligands. The gC1qR amino acid residues complementary in interacting with the respective ligand are provided, along with the exon the residues are found on. Along with binding to such ligands, this interaction with gC1qR is able to exert physiological effects to bring about relevant functions which can be modulated for therapeutic remedies.

Table 1.4: Functions of gC1qR

Function	Reference
gC1qR binds to HK and activates the coagulation cascade leading to the generation of the vasoactive peptide Bradykinin which causes vasodilation. Recently additional binding sites have been identified on gC1qR that are complementary to HK and are implicated to be targets for vascular permeability and inflammation.	Joseph et al 1994 Ghebrehwet et al 2012
gC1qR binds to endothelial to activate the classical pathway	Joseph et al 1999
gC1qR is able to bind to T and B lymphocytes and initiate an anti-proliferative response	Ghebrehwet et al 1990
Platelet gC1qR contains a binding site for staphylococcal protein A and plays a role in bacterial cell adhesion to sites of vascular injury and thrombosis.	Nguyen et al 2000
gC1qR forms a complex with C1q and DC-SIGN on the surface of iDCs to regulate DC differentiation	Hosszu et al 2012
gC1qR binding to platelets leads to the adhesion and activation of platelets	Peerschke et al 2003

1.12.3 gC1qR in Inflammation

The role of gC1qR in inflammation has been highlighted through its ability to bind plasma proteins such as C1q and HK (table 1.4). Studies have shown that both native and recombinant gC1qR are able to activate the classical pathway (Ghebrehwet et al, 1994). The binding site for gC1qR and IgG on C1q could potentially overlap since gC1qR incubated with serum caused a reduction in haemolysis when further challenged to

SRBCs. Structural data to support this has revealed that gC1qR interacts specifically within residues 155-164 of the A chain, which is also predicted to interact with IgG (Marques et al 1993). Given that gC1qR has complement inducing activity on endothelial cells and platelets (Peerschke et al 2004) appears to indicate that at sites of inflammation, gC1qR is a possible activator of the classical pathway.

Aside from the complement system, gC1qR is able to activate the Kallikrein/Kinin system (KKS) through its interaction with HK. HK interacts with gC1qR through its C terminal region on residues 204-218 (Joseph et al 1994) and is zinc dependent (Kaplan et al 2004). High affinity interaction between gC1qR and HK begins the assembly of the contact system where Kallikrein is formed and leads to the generation of the vasoactive peptide Bradykinin (BK). BK then exhibits its effects by binding to its B2 receptor (Regoli and Barabe, 1980) on the surface of endothelial cells. These cause vasodilatation and is enhanced through the production of vasodilators such as nitric oxide. BK's inflammatory properties are evident in the disease angioedema where it is responsible for the swelling observed.

1.12.4 DC-SIGN

Very recently, DC-SIGN (Dendritic Cell Specific ICAM3 grabbing Non Integrin) has been shown to be a ligand of C1q (Hosszu et al., 2012). DC-SIGN is a C-type lectin expressed on DCs (Geijtenbeek et al., 2000) that functions to mediate interactions between DCs and T-cells via ICAM3 (Intracellular adhesion molecule). The binding of DC-SIGN to its ligands such as ICAM-3, mannose and mannan is mediated through its carbohydrate recognition domain (CRD) and is calcium dependant (Curtis et al., 1992) which hypothesizes that DC-SIGN binding is carbohydrate dependant with high affinity to oligosaccharides which are present in both ICAM3 and gp120. C1q has been shown to co-localise with DC-SIGN and gC1qR on immature DC which has raised the idea that this complex of C1q with its receptor and ligand could be involved in DC differentiation through the binding of C1q with DC-SIGN signaling through the NF-kB pathway (Hosszu et al 2012). Evidence from Hosszu et al has shown that the binding of C1q to DC-SIGN has been inhibited by Ca⁺ and mannan suggesting that C1q binds to DC-SIGN via its Ca⁺ binding site through its globular domain. In light of this statement, it has been speculated that DC-SIGN shares the same binding site with C1q as IgG (Hosszu et al.,

2012) which puts forward the possibility of DC-SIGN-C1q being an initiator of the classical pathway. Additional significance of this interaction could be applied in an infection model where the interaction of these two proteins could somehow play a role in pathogen entry due to accumulating evidence of C1q receptors and ligands becoming key players in binding viral pathogens (Fausther-Bovendo et al., 2010) and infection transmission. DC-SIGN has also been shown to play a role in HIV infection where it binds to the HIV1 envelope protein gp120 (Masso, 2003). However DC-SIGN does not behave as a receptor on DC for viral entry, instead it acts as a reservoir by carrying DC cells with this viral infection through the lymphatics into the peripheral mucosa where it transmits HIV-1 to CD4+ T-cells leading to their infection and depletion as observed in the disease. This is evident on both immature and mature DC expressing DC-SIGN. The interaction of DC-SIGN with gp120 has been further elucidated by Sarker et al 2013 who has shown that the binding of DC-SIGN with gp120 leads to gp120 downregulating IL-6 release by Nef, another viral regulatory protein. The mechanism of this is brought upon by gp120 interacting with DC-SIGN which activates Ras-GTPase to induce Raf-1 which activates the NfKappaB pathway by translocating the p65 fragment of NfKappaB in the nucleus, and triggers IL-10 release. This IL-10 induction leads to the cytokine suppressor SOCS-3 being activated which initiates the downregulatory effect of IL-6 on Nef through gp120 on iDC. This clearly reflects on the DC-SIGN-gp120 induced signaling contributing to HIV pathogenesis.

1.12.5 DC-SIGN-R

DC-SIGN-R (DC-SIGN-Related) is a homolog of DC-SIGN that binds C1q (Kang et al., 2006). Both these C-type lectins share 77% sequence homology and binding is proposed to be calcium dependant. DC-SIGN-R is expressed on endothelium including liver sinusoidal, lymph node sinuses and placental capillary (Mitchell et al., 2001). Functions of DC-SIGN-R have been shown to lie with also binding ICAM-3 as well as gp120 to facilitate HIV viral infection (Bashirova et al., 2001). This C-type lectin has been previously noted to be the receptor for bacterial dextrans (Kang et al., 2003) and capsular pneumococcal polysaccharide (CPS) of *S. pneumoniae*. DC-SIGN-R binding to CPS has shown to lead to the proteolysis of C3 and binding of C3 opsonins to CPS (Kang et al 2006) which led to the discovery that SIGN-R binds directly to C1q upon interacting with the polysaccharide independent of an antibody. DC-SIGN-R is shown to

be highly expressed by spleen marginal zone macrophages (MZM) and lymph node macrophages (Leavy, 2006). It has very recently emerged that SIGN-R1 produced by MZM interact with C1q in the spleen and enhance apoptotic cell clearance through interacting with C1q and activating the SIGN-R mediated classical complement pathway. This newly discovered complement pathway uses macrophages expressed DC-SIGN-R to capture microbial polysaccharides in the spleen by directly binding to the bacterial surface which then allows C1q to engage with SIGN-R and activate the classical pathway (Prabagar et al., 2013). This illustrates that SIGN-R plays a role in apoptotic cell clearance and immune tolerance through mediating the classical pathway.

1.13 Collagen Binding receptors

1.13.1 Calreticulin

CRT is also referred to as the 'collectin receptor' (cC1qR) due to its ability to bind to various other collagenous domains of MBL (Mannan Binding Lectin) and Surfactant Protein A (SP-A) and SP-D (Malhotra et al., 1993). CRT is comprised of 3 domains, N, C and P (Figure 1.18), and studies have shown specific C1q binding domains in the N-terminal domain of CRT (Kishore et al., 1997). This domain essentially plays a role in interacting with glycoproteins and regulating the chaperone function of the protein. Alongside this, the N-terminal domain of CRT shows sequence similarity to IgG and its binding site has been shown to compete with IgG for C1q binding. Several studies have shown that such peptides inhibit complement activation by occupying the IgG binding site on C1q (Kovacs et al., 1998). As stated earlier, CRT and C1q has also been shown to play a role in apoptotic cell clearance via macrophages due to C1q being present on the surface of apoptotic cells and CRT linked to CD91 (Srivastava, 2002) at the monocyte cell surface (Gregory and Devitt, 2004; Kishore et al 1997) has shown that the globular head region of C1q also has the ability to bind to CRT and furthermore CRT deficiency showed an increase in C1q binding to apoptotic cells which revealed to be due to phosphatidylserine on apoptotic cells sharing the same binding site with CRT on C1q. This states an insight into the role of CRT and C1q in the removal of apoptotic cells through their involvement with PS which allows macrophages to connect with apoptotic cells. The chemotactic properties of gC1qR and CRT have also been observed by researchers who showed that DC cells treated with anti-gC1qR and anti-CRT reduced

chemotactic responses to C1q (Vegh et al., 2006). It was shown that the DC cells treated with anti-CRT entirely abolished DC chemotaxis, whereas anti-gC1qR treatment reduced cell migration highlighting that each of these receptors holds different chemotactic properties

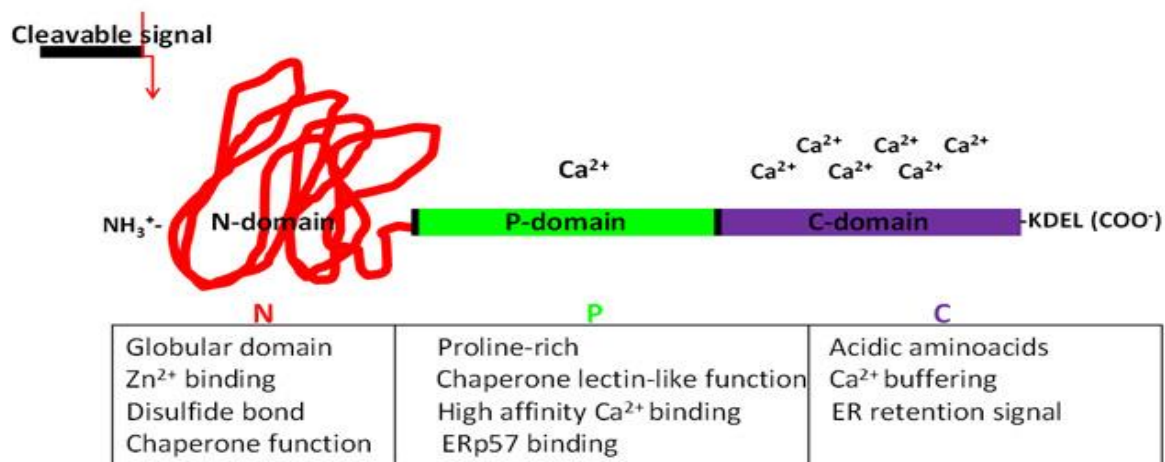


Figure 1.18: Structure of Calreticulin: CRT has a molecular weight of 55-60 kDa and is divided into 3 domains; the N-terminal (N), Proline- rich domain (P) and a C-terminal (C). The N terminal plays a role in interacting with glycoproteins and regulating the chaperone function of the proteins. It also binds Ca²⁺ with high and low affinity. The C- domain has high affinity for binding Ca²⁺ and helps regulate Ca²⁺ storage in the ER. The P-domain plays an important role in binding to ERp57 to aid disulphide exchange reactions in incorrect folded proteins in the ER (Mendlovic et al, 2011; Ellgaard et al., 2001).

Although CRT has been proposed to function as a receptor complex with CRT where CD91 acts as the phagocytic transmembrane molecule (Basu et al., 2001) and CRT engages directly with apoptotic cells, data has emerged to expose that C1q is capable of recognizing CD91 directly, independent of CRT (Duus et al., 2010). Experiments have shown that C1q binds to cells from a CD91 expressing monocytic cell line as well as monocytes from human blood with this binding corresponding with CD91 expression. Inhibition studies have reported that 45% inhibition was observed where CRT was used to interfere with C1q and CD91 binding, which suggests that only part of C1q is shared as a binding site for both these receptors (Duus et al., 2010). This identifies CD91 as a clear receptor for C1q and proposes that CD91 is a phagocytic receptor capable of binding to C1q and ingesting C1q bound material.

1.13.2 C1qRp

C1qRp is another putative receptor for C1q (Nepomuceno et al., 1997). This C1q receptor is a heavily glycosylated 97kDa protein expressed on myeloid cells, endothelial cells and platelets (Nepomuceno et al., 1999). C1qRp also known as CD93 has been found to bind to the collagen region of C1q as well as a number of other collectins such as Surfactant Protein A (SpA) and MBL. Structurally, C1qRp is composed of a C-type lectin domain, an array of epidermal growth factor like domains, a single transmembrane domain and a short cytoplasmic tail (Nepomuceno et al., 1999). Its role within C1q has been initially demonstrated to lie with C1q mediated enhancement of phagocytosis, as the binding of C1qRp and C1q resulted in increased phagocytic function with this enhancement dramatically reduced to 80% with anti C1qRp antibody. However, the functional role of C1q with this candidate receptor has raised a great deal of controversy as to how this molecule interacts with C1q on the cell surface to trigger biological responses. For instance, C1qRp-C1q interaction has been speculated by McGreal et al 2002 who showed under physiological conditions that cells infected with C1qRp failed to bind C1q. This puts forward the idea that C1qRp augments C1q mediated phagocytosis by monocytes as a co receptor as opposed to direct binding. However, this notion has been further challenged as Norsworthy et al 2004 have provided evidence using C1qRp deficient mice to indicate that CD93 deficiency in peritoneal macrophages has no impact on C1q mediated phagocytosis and instead illustrated an impaired ability to uptake apoptotic cells, thereby postulating a role for CD93 in the removal of dying cells. CD93 has also been hypothesized to play a role in cellular adhesion (McGreal and Gasque, 2002), due to its ability to bind vascular endothelial cells and its abundance of motifs common to other intracellular adhesion molecules. Nevertheless, further work based on this hypothesis (Norsworthy et al., 2004) states that CD93 deficiency has no effect in leading to cellular changes in distribution and adherence of peripheral blood cells. Quite recently, soluble CD93 expression has been known to be elevated in response to inflammation (Greenlee et al., 2009). Experiments undertaken by Greenlee et al 2009 to elucidate the molecular mechanism behind this CD93 dependant regulation of inflammation and any association with C1q have revealed using a thioglycollate induced peritonitis infection model that CD93 deficient mice after induction of peritonitis confirmed deregulation of C1q hemolytic activity which was correlated with increased leucocyte recruitment which

clearly highlights a connection between CD93 and the classical complement function of C1q.

1.13.3 $\alpha_2\beta_1$ Integrin

$\alpha_2\beta_1$ Integrin is a collagen binding receptor also capable of binding to laminins, decorins and several viruses (Zutter and Edelson, 2003). It is expressed on several cell types including epithelial cells, fibroblasts, endothelial cells and platelets. $\alpha_2\beta_1$ integrin has been suggested to hold a number of roles in inflammation (de Fougerolles et al., 2000), such as the deficiency of $\alpha_2\beta_1$ integrin in mice failing to elicit inflammatory responses to the Gram negative bacteria *Listeria monocytogenes* as well as high expression of this integrin expressed on peritoneal mast cells which are needed for inducing inflammatory reactions in response to infection in the peritoneal cavity (Echtenacher et al., 1996). In light of this statement C1q has been identified as a ligand for $\alpha_2\beta_1$ Integrin with this interaction specific to the α_2 integrin domain where C1q is shown to mediate PMC adhesion (Edelson et al., 2006). The capability of mast cells expressing $\alpha_2\beta_1$ Integrin to bind to C1q bound ICs and stimulate mast cell activation provides a favorable role for $\alpha_2\beta_1$ Integrin in modulating the immune response. Alongside this, it has been shown that $\alpha_2\beta_1$ Integrin-IC interaction is capable of providing signals to secrete cytokines by mast cells in response to various stimuli, and hypothesizes a role for $\alpha_2\beta_1$ Integrin in regulating autoimmune and allergic disorders. As $\alpha_2\beta_1$ Integrin serves as a collagen receptor on platelets, initial studies have identified this protein expressed on platelets involved to play a role in C1q binding (Peerschke and Ghebrehiwet, 1997b). However, antibody inhibition studies concluded a small decrease of 17% in platelet adherence to C1q which diminished the idea of $\alpha_2\beta_1$ Integrin involved in C1q- platelet adherence. The function of $\alpha_2\beta_1$ Integrin in the activation of mast cells could be explained through the indirect activation of the classical pathway. For instance, the binding of this receptor to C1q on ICs could trigger the complement cascade which leads to the deposition of C3b in turn generating C3a or C5a thereby activating mast cells.

1.13.4 Complement receptor 1 (CR1)

Complement Receptor 1 (CR1) is another protein identified as a C1q receptor specific to the CLR (Medof et al., 1982) and is expressed on eosinophils, neutrophils, monocytes, B and T lymphocytes and erythrocytes. Its role involving C1q lies within CR1 binding to C1q bound ICs and targeting them for clearance. Due to CR1 holding a major role on phagocytic cells in regulating the phagocytosis of complement opsonised pathogens (Gigli et al 1968), it is also capable of binding to the opsonins C3b and C4b (Tas et al., 1999) which attach onto ICs to enhance their clearance. The cellular function of CR1 widely differs depending upon the type of cell it is expressed on. For example it has recently been found that CR1 expression levels hold implications in RA (Kremlitzka et al., 2013) due to its downregulation on B cells observed in the disease. However despite this low expression of CR1, it has been shown to have no effect on the inhibitory function of B cells as data has illustrated CR1 clustering by its ligand to result in major inhibition of B cell proliferation in correlation to what is seen in healthy individuals which raises the point that CR1 hold alternate roles as a possible therapeutic target in arthritis. CR1 has also been shown to inhibit the differentiation of B cells to plasmoblasts, as well as inhibit their antibody production highlighting a role in B cell activation. The role of this complement receptor expressed on erythrocytes has been revealed to bind to C1q and mediate erythrocyte adhesion (Tas et al., 1999). Moreover, experimental evidence on erythrocytes has confirmed that CR1 plays the role of an immune adherence receptor by being able to bind to the opsonic ligands C3b and C4b and mediate adhesion. The fact that C1q has been shown to enhance C4b mediated adhesion to CR1 is of great beneficiary in cases with lower levels of C4b. interestingly, CR1 dependant phagocytosis of complement opsonised pathogens is decreased in SLE patients (Mir et al., 1988) which suggests that this impaired function of CR1 on phagocytes could somehow give rise to these autoimmune disease. Although C1q also opsonises ICs (IC), research has shown that CR1 failed to remove IC in patients deficient for C3b and C4b which emphasizes that the interaction between C1q and this receptor is not essential in clearing of IC

1.13.5 C1qR₀₂

C1qR₀₂ is a C1q receptor that has been identified to trigger superoxide in polymorphonuclear leucocytes (Tenner, 1998). The generation of superoxide is a protective mechanism produced by immune cells to help destroy pathogens. The activation of superoxide leads to degranulation of the cell due to secretion of proteolytic enzymes from the granules. However, C1q mediated activation of O₂⁻ does not lead to degranulation of the leucocyte. Alongside this, C1q regulated respiratory burst does not require increased cell adherence, a feature which is accompanied by oxidative burst (Goodman et al., 1995). This holds implications in diseases such as SLE where the non-specific host tissue damage could be due to activation of the oxide produced by C1q (Schur, 1982). Evidence for the C1qR₀₂ specificity for C1q comes from antibodies that bind C1qRp on neutrophils failing to inhibit C1q induced production of O₂ by neutrophils (Guan et al., 1994). Structural studies within this C1q and this receptor have shown that the C1qR₀₂ binding site on C1q required triple helical secondary structure in for functional activity to take place (Ruiz et al., 1995). In addition to this, the kink region of C1q is also hypothesized to be essential for functional binding between C1q and C1qR₀₂. The active site on C1q accountable for generating O₂ production has been recognized as amino acids 42-61 of the C chain of the CLR.

In this thesis we have addressed the nature of interaction between gC1qR and some of its known biologically relevant ligands. Thus,

Chapter 3 examines the interaction between recombinant gC1qR and recombinant globular head modules of human C1q. In order to further characterize the interaction, a number of substitution point mutations were examined for their affinity to gC1qR. Since C1q expression is upregulated in adherent monocytes, we also examined concomitant expression of C1q and gC1qR in adherent monocytes with and without challenge with a pro inflammatory stimuli such as lipopolysaccharide (LPS). C1q is known to have an anti-proliferative effect, thus, the ability of globular head modules and gC1qR to modulate C1q functions on mitogen stimulated PBMCs is also a subject of investigation and discussion.

Chapter 4 examines interaction between gC1qR and its newly discovered adaptor molecule on the cell surface DC-SIGN whose interaction is likely to have profound implications on HIV-1 infection of Dendritic Cells. Using recombinant forms of monomeric and tetrameric DC-SIGN and SIGN-R we have extensively characterized the interaction between the tripartite interaction between globular head modules, gC1qR and DC-SIGN. These studies are now ripe for further understanding the modulation of HIV infection which is currently underway.

Chapter 5 examines gC1qR ability to act as pattern recognition receptor for HIV-1 gp41 and HCV core protein. The structure function relationships between gC1qR and its viral ligands have been extensively studied using recombinant proteins and synthetic peptides.

Chapter 6 identifies the ability of gC1qR to bind to endothelial cells via fibrinogen and upregulate the surface expression of bradykinin 1 receptor using a range of binding and microscopic experiments, this chapter provides an excellent model of how gC1qR can link the contact system that is KKS.

CHAPTER 2:
GENERAL MATERIALS AND METHODS

2.1 Purification of Human C1q

C1 was purified from freshly-thawed serum. Briefly, freshly thawed serum was made 5mM EDTA and centrifuged to remove aggregated lipids. The serum was then incubated with non-immune IgG coupled to CnBr-activated sepharose (GE Healthcare, UK) for 1 hour at 4degrees. The plasma with IgG-sepharose was filtered through a scintered glass funnel, ensuring that no air bubbles are formed. The C1q-bound sepharose is then washed extensively with 10mM HEPES, 140mM NaCl, 0.5mM EDTA, pH 7.0 and C1q is eluted with CAPS buffer (100mM CAPS, 1M NaCl, 0.5mM EDTA, pH 11.0). The eluted C1q is then passed through a HiTrap ProteinG column (PierceNet, USA) to remove IgG contaminants and dialysed against the washing buffer.

2.2 Constructs expressing Wild Type and substitution mutants of ghA, ghB and ghC modules of C1q

The C-terminal globular region of human C1q A (ghA), B (ghB) and C (ghC) chains were expressed as maltose binding fusion proteins using the expression vector pMal-c2 (figure 2.1) as described by earlier (Kishore et al 2003). Each mutant has been altered by substituting a single amino acid involving either Arginine (R) to Alanine (A), Glutamate (E), or Glutamine (Q) substitutions, or Histidine (H) to Alanine (A) or Aspartate (D) substitutions, and Leucine (L) to Glutamate (E). The following mutants were purified: ghA mutants were Arg^{162E}, Arg^{162A}, ghB mutants were Arg^{114Q}, Tyr^{175L}, Arg^{114A}, Arg^{129A}, Arg^{129E}, Arg^{163E}, Arg^{163A} and His^{117D}. ghC mutants were Arg^{156Q}, Arg^{156E}, His^{101A} and Leu^{170E}. The generation of various mutants has been described previously by Kojouharova et al 2004

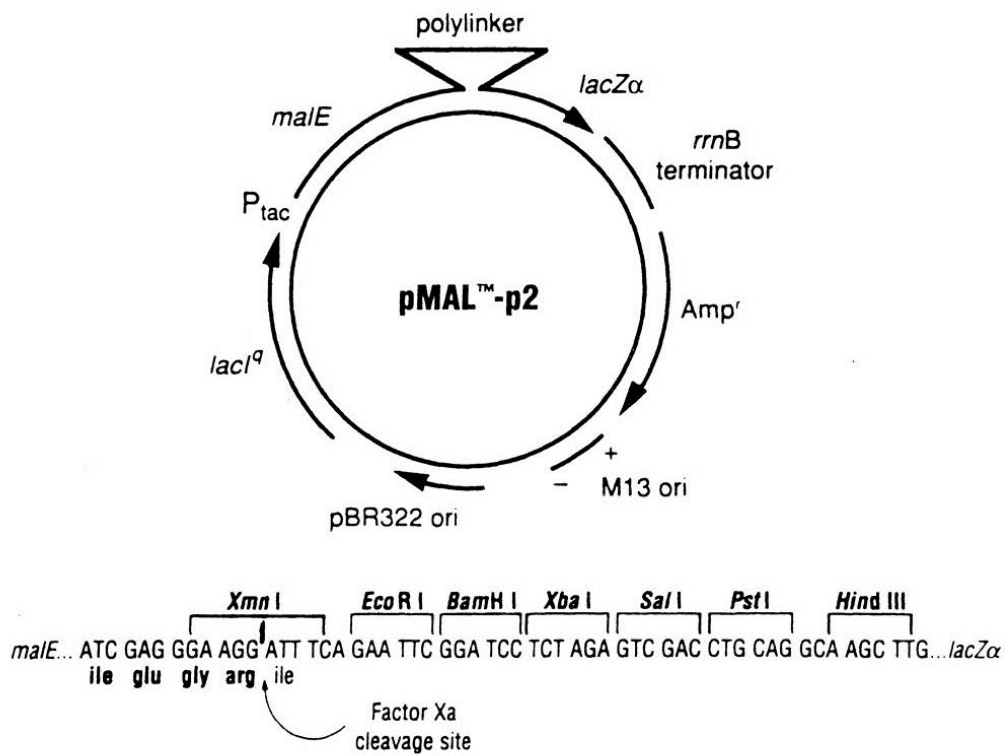


Figure 2.1: pMalc vector:

The pMal-c2 vector was used to express the ghA, ghB and ghC globular head substitution fusion proteins. The gene was cloned downstream to the *malE* gene of *E.coli* which codes for maltose-binding protein (MBP) to results in a MBP fusion protein (Maina et al., 1988)

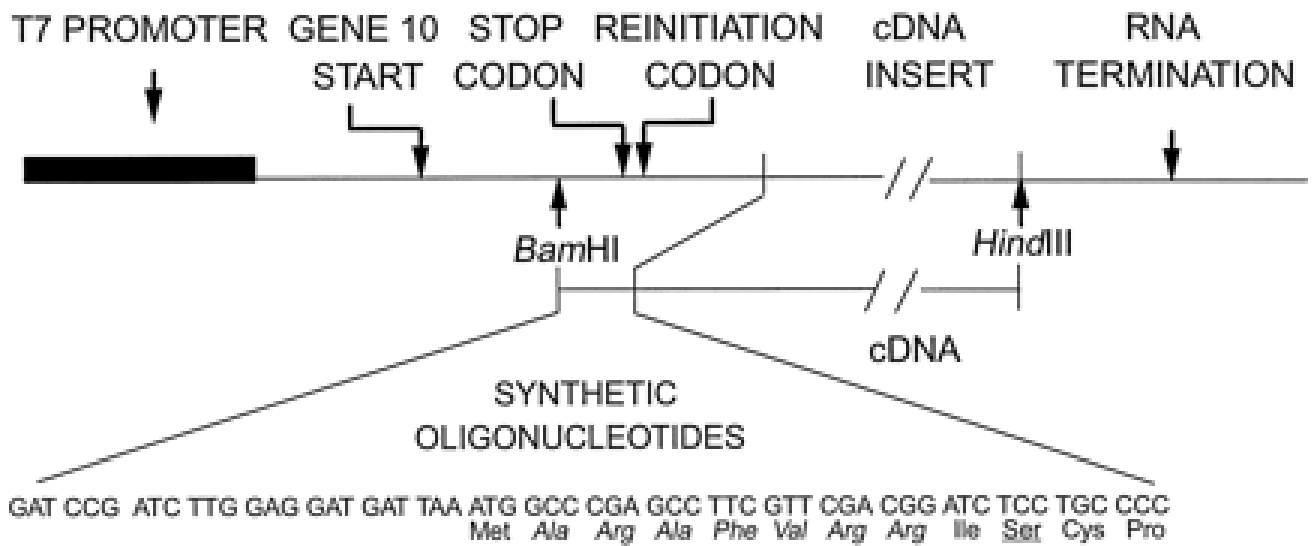


Figure 2.2: pT5t vector: The cDNA for DC-SIGN tetramer, DC-SIGN monomer, SIGN-R tetramer and SIGN-R monomer was inserted in the pT5t vector

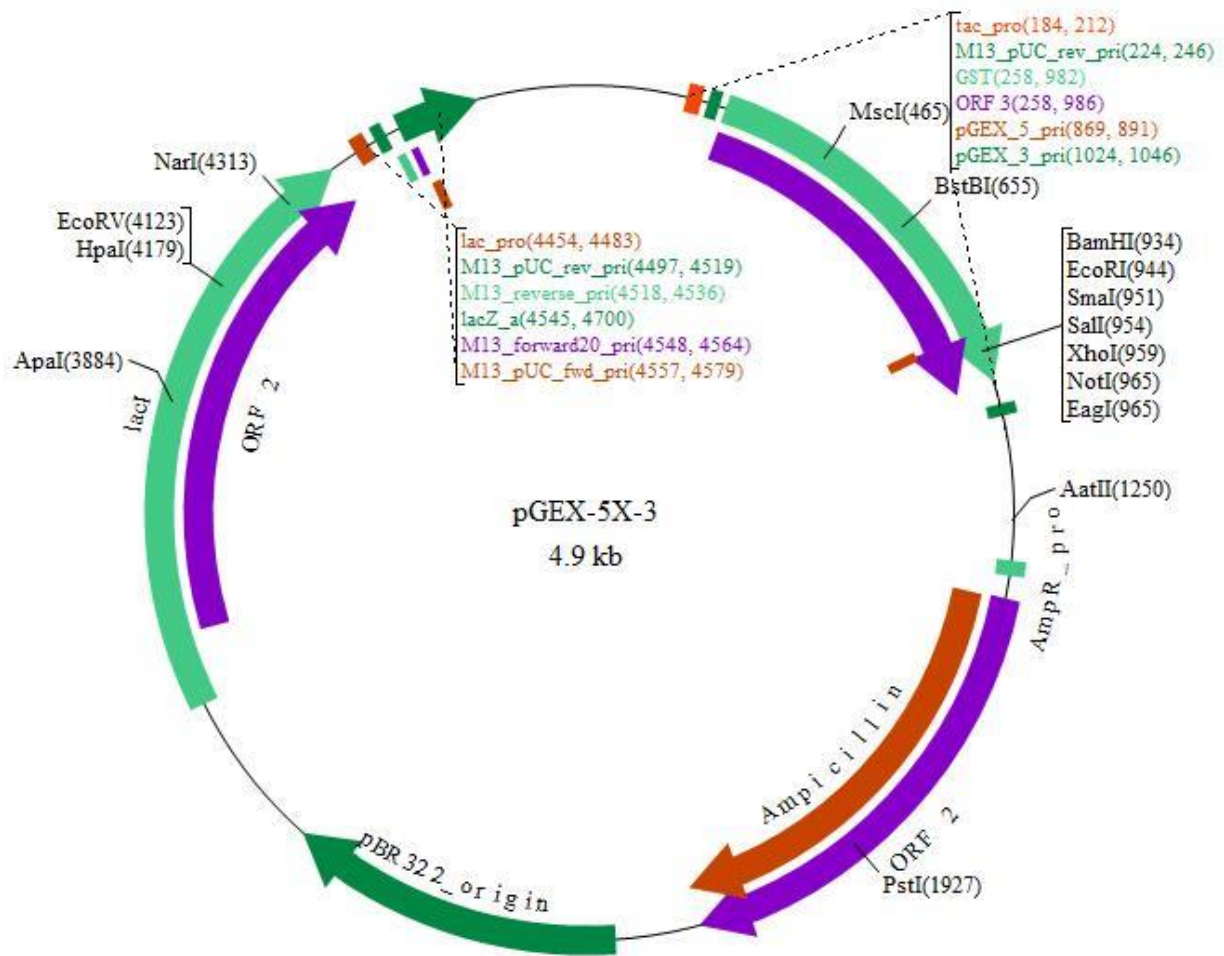


Figure 2.3: pGEX vector: Map of the glutathione S-transferase fusion vector used for generating the gC1qR deletion mutants fused to GST.

2.3 Bacterial Transformation

Various expression constructs used for making the recombinant proteins were transformed in BL21, the host strain of *E. coli* and selected on Ampicillin (100µg/ml) LB agar plates, with the exception of the transformant expressing the monomeric form of SIGN-R, which was selected for kanamycin resistant (50µg/ml).

A single colony of BL21 (DE3) was inoculated in 5ml of LB (Lauria Broth) medium and grown overnight in a 37° shaker. The next morning 500µl of this overnight culture was inoculated in 25ml LB and grown at 37°C, A_{600} was read every hour until the cells reached a value of 0.3. The cells were then pelleted by centrifugation at 3000rpm for 10 minutes and the supernatant discarded. The cell pellet was then gently re-suspended in 12.5ml of 0.1M $CaCl_2$. This was then left on ice for an hour and centrifuged again. The cells were resuspended in 2ml of ice cold 0.1M $CaCl_2$ (Competent cells). 200µl of competent cells were added to an eppendorf tube containing 2µl of the plasmid. This mixture was left on ice for an hour. The mixture was then heat shocked at 42°C for 90 seconds. 800µl of LB medium was added to the mix and left in the 37°C incubator for 40 minutes. 150µl of this culture was then plated out on the antibiotic containing plate (LB-Ampicillin or LB-Kanamycin) and left to grow overnight at 37°C.

2.4 Pilot scale testing for the recombinant Protein Expression using IPTG

A single transformed colony was selected from its transformed plate and incubated in 5µl of its resistant antibiotic and 5ml of LB overnight in a 37°C shaker. The next morning, 200µl of the overnight culture was added to 10ml of LB with 10µl of its antibiotic and left to grow in the 37°C shaker until it reached a reading of 0.6. 1ml sample was then removed to test for uninduced expression and IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the sample and left in the 37°C shaker for 3 hours. Once induction time was over, 1 ml sample was collected to assess the induction of the expression. Both uninduced and induced samples were spun down at 13,000rpm for 10 minutes using a micro-centrifuge and the supernatant was discarded. 100µl of treatment buffer was added to the samples and they were heated at 100°C for 10 minutes. Expression for uninduced and induced samples was tested on an SDS-PAGE gel. Once the best colony for expression was established the next step was to upscale it.

2.5 Upscale of the wild-type and substitution mutants of the globular regions of C1q A, B and C chains (ghA, ghB and ghC, respectively)

A single colony was inoculated individually in 5ml of LB and 5 μ l of Ampicillin and left to grow overnight in a shaking 37°C shaker. The next morning, the overnight inoculum was transferred into 200ml of LB with 200 μ l of ampicillin and left to grow in the 37°C for 2-3 hours until the cells reached an OD (600 nm) of 0.6. 1ml of the culture was transferred into an eppendorf tube to use as an uninduced sample. The rest of the culture was induced with 0.5mM IPTG and both un-induced and induced samples were further incubated for 3 hours in a 37°C shaking incubator. After induction was complete, 1ml of the induced sample was transferred into an eppendorf tube and along with the un-induced sample, the samples were centrifuged at 13,000rpm for 10 minutes and the supernatant discarded. The pellet was resuspended in 100 μ l of treatment buffer and heated on a heat block at 100°C for 10 minutes. Expression was tested on a 10% SDS-PAGE gel by loading 15 μ l of each sample and running the gel at 120v. The 200ml culture was harvested by centrifugation at 4500rpm for 10 minutes and the pellets were stored at -20°C until required for further processing.

2.6 Purification of the wild-type and substitution mutants of the globular regions of C1q A, B and C chains (ghA, ghB and ghC, respectively)

The cell pellet obtained after IPTG induction was thawed at 37°C and resuspended in lysis buffer (containing 0.1g lysozyme, 20mM Tris pH 8.0, 0.5M NaCl, 1mM EDTA, 0.2% Tween 20, 5% glycerol and 0.1mM PMSF). This was left on a rocker in the cold room for 1 hour. Following this, the sample was sonicated on ice, (8 cycles at 30 seconds each with 2 minute gaps where the sample was placed on ice). The samples were then aliquoted into eppendorf tubes and spun down at 13,000 for 15 minutes. The supernatant was collected and run on a 15% SDS gel to confirm the presence of target protein in the soluble fraction. ghA, ghB and ghC and its mutants were purified by running them through Amylose resin columns with a bed volume of 30ml. MBP being a maltose binding protein, therefore an Amylose resin was used which would bind the MBP fused to the globular head from the unpurified soluble fraction of the total *E.Coli* extract supernatant.

2.7 Preparation of Amylose Resin and DEAE Sepharose columns

Amylose Resin was purchased from New England Biolabs cat number: E8021L and DEAE Sepharose from GE Healthcare Life Sciences cat number: 17-0709-01. 5ml of the resin was washed in autoclaved dH₂O in a beaker by gently shaking and discarding the water. This was repeated 3 times. The resin was then packed into a disposable plastic column with a polyethylene filter disc (Pierce, Cat no: 299920) allowing the water to pass and leaving the resin intact in the column.

2.8 Purification using Amylose Resin Column

The columns were first washed with 0.1% SDS three times, autoclaved dH₂O three times and buffer I (20mM Tris pH 8.0, 100mM NaCl, 0.2% Tween 20, 1mM EDTA and 5% glycerol) three times. 30ml of Buffer I was loaded on the column following the supernatant of the protein which was mixed with Buffer I on a ratio of 1:5. This followed two 30ml bed volumes of buffer II (250ml of buffer I (without Tween 20) made up to a litre with water). The column was then eluted with 10mM maltose (100ml of buffer II with 10mM Maltose) and collected in 15 fractions of 2ml eppendorf tubes and frozen at -20°. As MBP has its highest affinity for maltose, it would compete with the amylose resin and attach to Maltose, enabling the fusion protein to be eluted from the column where it was collected.

2.9 Cloning, Expression and Purification of human gC1qR

Recombinant mature gC1qR consisting of sequences 74-282 were expressed in *E.coli* BL21. Colonies selected from the transformed cells were inoculated in 5ml LB and 5µl of Ampicillin overnight at 37°C. The next morning, the overnight inoculum was added in 250ml of LB and 250µl of Ampicillin and grown at 37°C until an OD of 0.6 was reached. The cell culture was then induced with 100µg/ml of IPTG. Following a further incubation for 3 hours, the cell culture was spun down (4500rpm for 15minutes). The cell pellet was treated with lysis buffer (0.5M NaCl, 20mM Tris pH 8.0, 1mM EDTA, 0.2% Tween, 5% glycerol, 0.1g lysozyme) and incubated for 1 hour at +4 shaking. The cell lysate was sonicated for 10 cycles for 30 seconds with 2 minute intervals. Following this the lysate was spun down at 13000rpm for 15mins and the supernatant was collected.

2.10 Ion Exchange of gC1qR

The supernatant was dialysed using dialysis tubing for 2 hours against 1 litre of 100mM NaCl and 20mM Tris at +4°C. The dialysed protein was subjected to ion exchange using a 5ml DEAE Sepharose column. The column was first equilibrated by washing with 5 bed volumes of dialysis buffer. Next the protein sample was passed through the column. Purified gC1qR was eluted at a peak concentration of 0.45M NaCl and collected in 1ml fractions. Each sample was then run on a 15% SDS-PAGE gel to assess purity and samples were pooled to give a final concentration.

2.11 Construction of gC1qR deletion mutants

The gC1qR deletion mutants were expressed and purified using the pGEX vector (figure 2.3) and kindly provided to us by Ghebrehiwet et al. The rationale was generating these mutants was to confirm previously identified binding sites or to discover new ones. The deletion sequences were chosen by deleting those sequences predicted to have either a membrane attachment or ligand binding site. The residues to be deleted were selected by analysing the crystal structure and identifying residues involved in ligand binding. The deletions were generated using complementary sense and antisense primers that bridge the deleted region. Maximum of mutagenesis was achieved by bringing the melting temperature on each side of the deleted sequence to 60°C. Primers were extended using Pfu Turbo polymerase and the mutant constructs were enriched by digestion of the methylated template with Dp1 and transformed into *E.coli*. A single residue mutation was performed on Trp233 which is the only domain that projects out on the solution phase side of gC1qR in the crystal structure. Encoded by the codon TGG, this residue was mutated by a single base change to GGG (Gly) using double strand mutagenesis. Due to its conspicuous projection, this site is considered to be a wide target for plasma or pathogen associated ligands.

2.12 SDS-PAGE

SDS-PAGE was used to separate protein samples according to their molecular weight. The size of the protein was estimated using a molecular weight marker. The gel comprised of two parts, a stacking and a resolving gel. The composition of the stacking gel was the same regardless of the percentage of the gel. A separating comb for samples

to be loaded into these wells was placed in the stacking gel as soon as it was loaded above the resolving gel. After the gel was polymerized, it was placed in a tank and 1 X running buffer was poured into the tank over the gel and the separating comb was removed. The protein samples were mixed with an equal volume of bromophenol blue loading dye and denatured by heating at 100°C for 10 minutes before being briefly centrifuged prior to loading in the wells. A molecular weight protein marker was also loaded at approximately 3 µl and the gels were run at 120V for 1.5 hours. The gel was then stained with staining solution (50% Methanol, 40% acetic acid with 0.1% Coomassie blue). This was left on a rotating rocker for 4hours minimum for proteins to incorporate the dye before the de-staining process. After destaining the gel, only the proteins stained with Coomassie blue were visible as well as the protein marker to estimate the size of the protein. The gel images were taken using the Molecular Imager Gel Doc (Peqlab, Sarisbury Green, UK).

For most purposes a 12% v/v SDS-PAGE gel was made by making a resolving gel followed by a stacking gel. The 10ml resolving gel was made up using the following reagents:

Table 2.1: Components of Resolving gel

Component (12% Resolving Gel)	Volume
dH₂O	3.3ml
30% Acrylamide	4ml
1.5M Tris-HCL, pH8.8	2.5ml
10% sodium dodecyl sulfate (SDS)	100µl
Ammonium persulfate (APS)	100µl
TEMED	15 µl

The resolving gel was left to polymerise at room temperature. Once polymerised the stacking gel was made and loaded above the resolving gel with a separating comb embedded in it. The stacking gel composition is the same for all percentage gels and is composed of:

Table 2.2: Components of stacking gel

Component (Stacking Gel)	Volume
dH₂O	3.4ml
30% Acrylamide	830µl
1.5M Tris-HCL, pH8.8	630µl
10% sodium dodecyl sulfate (SDS)	50µl
Ammonium persulfate (APS)	50µl
TEMED	15 µl

2.13 BCA Protein Estimation

The Pierce BCA Protein was used to quantitate the protein. This assay is based on a colorimetric method. Firstly, diluted bovine serum albumin (BSA) standards and BCA working reagent was prepared. The working reagent was made by mixing 50 parts of BCA reagent A with 1 part BCA Reagent B (50:1). 0.1 ml of each standard and unknown sample replicate was pipetted into a test tube, and 2 ml of the working reagent was added to each tube followed by thorough mixing. The tubes were then incubated at 37°C for 30 minutes and then brought to room temperature. The absorbance of each tube was measured at 562nm and a standard curve was prepared by plotting the average of the OD values for each BSA standard versus its concentration in µg/ml. The protein concentration of each sample was then determined by using the plotted standard graph (figure 2.5).

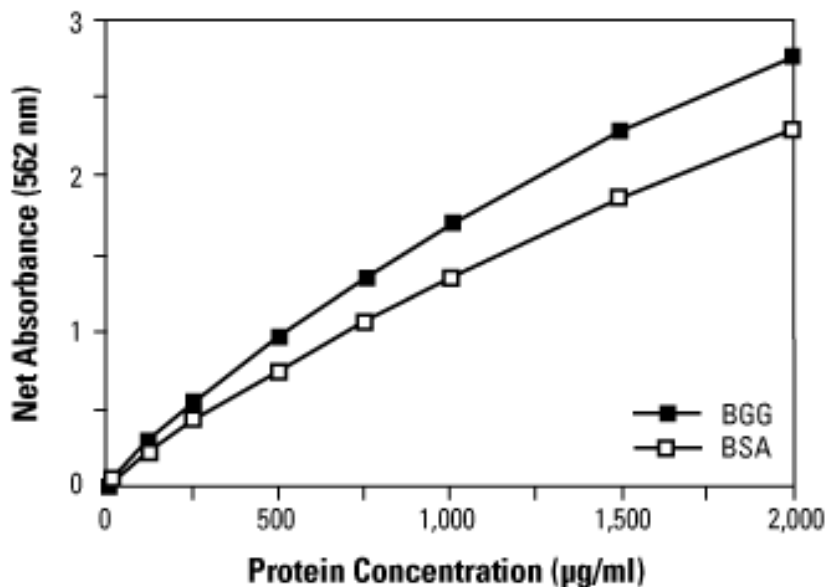


Figure 2.4: BCA standard curve: Typical standard curves for bovine serum albumin (BSA) and bovine gamma globulin (BGG) in the BCA Protein Assay.

2.14 ELISA (Enzyme Linked Immuno Sensitive Assay)

2.14.1 Direct Binding ELISA

A 96 well plate was used for all ELISAs and each sample was tested in duplicates. Poly-L-lysine wells were coated with different concentration of protein in serial dilutions ($\mu\text{g}/\text{well}$) in $100\mu\text{l}/\text{well}$ carbonate/bicarbonate buffer pH 9.3 and left overnight at 4°C . Following morning the buffer and unbound proteins were discarded and wells were blocked with $100\mu\text{l}$ of 2% w/v BSA in PBS for 2h at 37°C . The wells were then washed three times using PBS + 0.05%v/w Tween 20. The desired protein was diluted in Calcium buffer (10mM Tris-HCL pH 8, 5mM CaCl_2 , 50mM NaCl) and added at a constant concentration ($\mu\text{g}/\text{well}$) of $100\mu\text{l}$. The microtitre plate was incubated at 37°C for 1.5 hours and 4°C for 1.5 hours. Next, the microtitre wells were again washed three times with PBS + 0.05% Tween and $100\mu\text{l}$ per well of polyclonal antibody (pAb) or monoclonal antibody (mAb) in PBS at the relevant concentration was added and incubated for a further 1 hour. Three washes were then repeated and $100\mu\text{l}$ of the secondary antibody, either rabbit anti mouse IgG-Horseradish peroxidase (HRP) binds to mAb) or Protein A (PA)-HRP (binds to pAb) at a dilution of 1:5000 in PBS was added to each well. Wells were then washed again. Finally, the reaction was developed using

OPD substrate which reacted with the HRP conjugated protein A and optical density was determined under a plate reader at 450 nm. The OPD tablets were diluted in 10 ml of water and 100 µl of OPD was added to each well to develop a colour from the enzyme-substrate reaction. The optical density of the plate was read under a plate reader at 450 nm.

2.14.2 Competitive ELISA

The protein of interest was coated on microtitre wells at a constant concentration (µg/well) in bi-carbonate/carbonate buffer pH 9.6 and left overnight at 4°C. The following morning blocked was performed as described above. The microtitre wells were then washed three times in PBS + 0.05% Tween. The two competitors of proteins were added together in a total volume of 100µl buffer per well with one protein at a constant concentration and the other competitor at a range of serial dilutions. The plate was then incubated for 1.5 hours at 37°C and 1.5 hours at 4°C. Following repeated wash, the protein added at a constant concentration was probed using its primary antibody approximately diluted in PBS, and then incubated for an additional hour at 37°C. The secondary antibody conjugated to HRP was added in PBS to the wells and incubated for 1 hour at 37°C. Following repeated washing, the plate was read at 492 nm after addition of OPD.

2.15 Far Western Blot

A SDS-PAGE was prepared using the biorad rainbow marker (Cat no #161-0375). The protein(s) were diluted in an equal amount of treatment buffer and heated for 10 minutes at 100°C. Following a quick centrifuge the sample(s) were loaded into the well(s) and the gel was run at 120V.

After the protein sample had passed through the gel for separation, it was ready to be transferred to PVDF membrane. Initially, The gel and western blot material consisting of 4x 2.5mm Whatman paper, 2x fibropads and PVDF membrane were equilibrated in transfer buffer (80% v/w Methanol, 5.8 g Tris Base, 2.9 g Glycine and 0.37 g SDS) for 15 minutes. The apparatus was set up in the following order: black side- fibropad-whatman paper-gel-nitrocellulose membrane-Whatman paper-fibropad-white side, in the transfer buffer. As each layer was applied, a serological tip was used to smooth out all air bubbles which would alter sufficient transfer of the bands. The western blot

sandwich was then placed in the holder ensuring the black side was facing the black, and once in the tank, the transfer buffer was poured and the lid was secured well. The transfer took place at 320mA for 1 hour with a magnetic stirrer and ice pack in place to prevent overheating.

Once the protein transfer was finished, the PVDF membrane was carefully transferred into a plastic cassette using tweezers and blocked for 1 hour at room temperature in the blocking solution made up of 3% Non-fat milk powder in PBS. Following this, the membrane was incubated overnight at 4°C in the probing protein in the binding buffer (50mM Tris-HCL pH 8.0, 100mM NaCl, 5mM CaCl₂). The membrane was then washed 5x in PBS + 0.02% v/w Tween 20 for 10 minutes each to ensure removal of any non specific protein binding. Next, the primary antibody was added at the relevant concentration in 10ml of 1% non-fat milk powder in PBS and incubated for 2 hours at 37°C. The membrane was then washed as described above and the secondary antibody conjugated to HRP was diluted in 10ml 1% v/w non fat milk powder in PBS at the relevant concentration. This was incubated for 1 hour at room temperature. Repeated washes followed this and the membrane was developed using diaminobenzidine peroxidase (DAB).

2.16 Plasmid Preparation

Competent cells were prepared as mentioned above using TOPO 10 strain of *E. coli*. The plasmid required to be made was transformed in TOP010 as described in 2.3. The following evening a single transformed colony was inoculated in 5ml LB and left to grow shaking at 37°C overnight. The next morning 3 ml of the overnight culture was spun at 13,000 for 2 minutes. Quigen kit (Cat no: 27104) was used to extract plasmid. The supernatant was discarded and 250µl of Resuspension buffer (P1) was added, followed by 250µl of lysis buffer (P2) and left at room temperature for 2 minutes. Next, 350µl of neutralization buffer was added and the sample was mixed immediately by repeatedly and gently inverting the eppendorf tube upside down. The sample was then left on ice for 5-10 minutes. Following this, the eppendorf tube was spun at 13,000 for 10 minutes and the supernatant was carefully transferred onto the mini spin column. Once the sample had flown through, the column was spun at 13,000 for 1 minute and the flow through was discarded as the DNA would have been bound to the column. Next, 500µl of

binding buffer was added to the column and spun at 13,000 for 1 minute, the flow through was discarded. 750µl of wash buffer was then added to the column and spun at 13,000 for 1 minute. The flowthrough was discarded and the column was spun for another 1 minute to ensure elimination of all the buffers. The column was then transferred onto another eppendorf tube and 50µl of elution buffer was added and left to flowthrough for 2-3 minutes. Following this, the column was spun at 13,000 for 10minutes and the supernatant (plasmid) was collected and frozen at -20.

2.17 Agarose Gel Preparation

To ensure the correct plasmid was extracted, the sample was run on an agarose gel. Firstly, 0.5g of agarose was weighed out and added to 75ml of 1x TBE (Tris Boric acid, EDTA) buffer which was made by diluting 100ml of the 10x stock (108g Tris base, 55g Boric Acid, 7.5g EDTA) with 900 ml of dH₂O and heated until the agarose was dissolved. The solution was then left to cool and the agarose gel apparatus was prepared by taping both sides of the tank and positioning the comb in the slots. Once the agarose TBE solution had cooled, 7µl of Ethidium Bromide was added and the solution was mixed to dissolve and poured into the casting tray. Additional TBE buffer was added into the tray until the gel was immersed. 5µl of Peqlab DNA ladder (Cat no: 25-2000) was loaded into one of the wells and 5µl of the plasmid was mixed with 5µl of DNA loading buffer and loaded into another well and left to run at 70V for 1 hour. The amount of plasmid DNA was estimated at OD₂₆₀ using the nanodrop.

2.18 Ion Exchange

An anion column which is a positively charged solid support, DEAE Sepharose was used to further purify negatively charged proteins. 5 ml of DEAE sepharose (GE Healthcare Life Sciences, Cat no:17-0709-01) resin was packed in a column and washed with three bed volumes of distilled H₂O. The protein subjected to ion exchange was dialysed overnight in 20mM Tris pH7.5 and 100mM NaCl. The column was equilibrated with three bed volumes of dialysis buffer. Next, the protein sample was passed through the column. If the peak molarity of NaCl required to elute the protein was initially known, this was collected in 10ml of 1ml fractions. 30µl was removed to run on a SDS-PAGE gel and the fractions were frozen at -20. If the elution peak was not known, only 2ml of

protein sample was initially passed and a NaCl gradient was performed which involved using different NaCl concentrations, 100mM, 200mM, 300mM, 400mM, 500mM, 600mM, 700mM and 800mM. 10ml of each concentration of NaCl was passed through the column and collected. 30µl of each NaCl concentration was mixed with 30µl of treatment buffer and run on a gel to see at which concentration the protein elutes at. Once the concentration was known, ion exchange was repeated using the total protein sample and eluted at the relevant concentration in 1ml fractions. Samples were frozen at -20.

2.19 Biotinylation of Proteins

2.2mg of biotin (Sigma, Cat no: B4501-100MG) was weighed out and dissolved into an eppendorf tube containing 400µl of dH₂O and dissolved. 3µg of the biotin mixture was added per 100µg of the protein and incubated for 1 hour at room temperature. Next, the protein was dialysed against 1 litre of PBS. To confirm biotinylation of the protein, 1µg/well of the protein was coated on microtitre wells and probed with Streptavidin-HRP.

2.20 Limulus Amebocyte Lysate (LAL) assay

The LAL assay is a test that detects the gram negative bacterial endotoxin LPS within protein samples. By adding the LAL reagent, the LPS level is identified by the sample undergoing a colorimetric change to yellow. The absorbance is read at a wavelength of 405nm using a spectrophotometre and the endotoxin level determined using the standard curve shown in figure 2.5.

Endotoxin Standard Curve graph

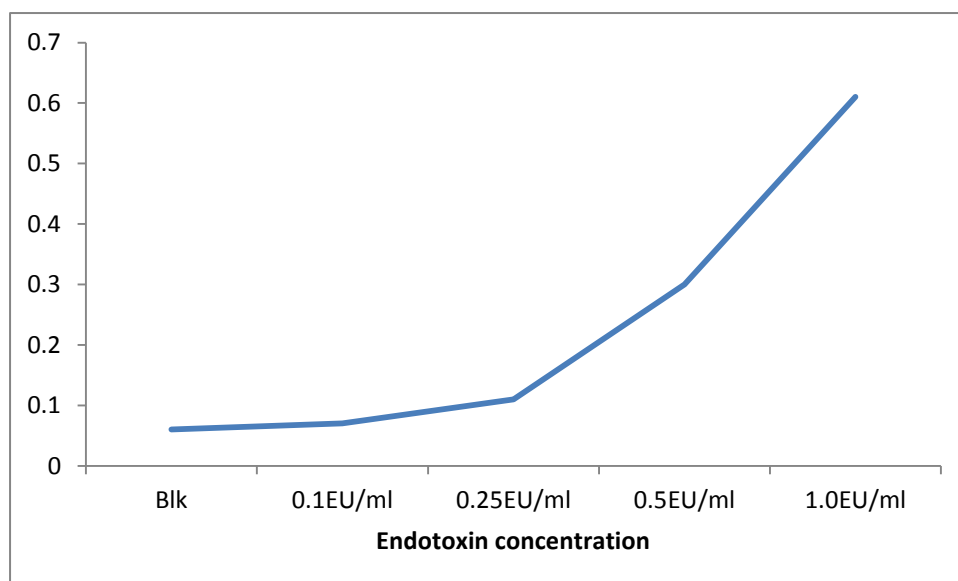


Figure 2.5: Endotoxin standard curve: Standard graph to show levels of endotoxin concentration provided in the kit. The endotoxin level in the protein sample can be calculated according to the graph.

The LAL reagent contains lyophilized lysate from *Limulus polyphemus*. LPS present in the protein sample catalyses a pro enzyme in the Limulus Amebocyte Lysate to an active enzyme. The activated enzyme then catalyses splitting pNA. Once the substrate is released it is measured spectrophotometrically against a linear gradient of a standard endotoxic graph (figure 2.5).

Each standard contains approximately 15-40 Endotixin Units (EU) of lyophilized endotoxin. Once vial is reconstituted with 1ml of LAL reagent water at room temperature (to yield a concentration stated on the manual, eg, 24 EU.ml) and vortexed for 15 minutes to detach the endotocin from the glass. The substrate used is approximated 7mg of lyopholised substrate which is reconstituted in 6.5ml of LAL reagent water to yield a concentration of ~2mM. To prepare a set of standards, the reconstituted endotoxin vials were vortexed for 1 minute and then diluted to 1EU/ml. 0.5ml of this was transferred into another glass vial and diluted with 0.5ml LAL reagent water to halve the concentration of EU/ml. 5-10 standards were prepared and vortexed for 1 minute before use. The samples were pipetted from tube to tube at the same rate to allow the same time for reaction. 50 μ l of the standard was mixed with 50 μ l of LAL reagent.

2.21 Removal of endotoxin containments from recombinant proteins purified from *E.coli*

The removal of endotoxins from recombinant proteins is important to prevent interference and false positive signals in further studies caused by LPS. LPS is found on the outer cell membrane of gram negative bacteria. Thermo Scientific Pierce High Capacity endotoxin removal resin in spin columns were used for the removal of LPS. The resin contained cellulose beads with covalently attached poly-L-lysine which has a high affinity for endotoxins. The binding capacity is 2 million EU per ml, reducing endotoxin levels by 99% and should typically result with a final endotoxin concentration below 5 EU/ml which is of an acceptable range.

The use of the columns was followed as instructed by the manual provided. The column was briefly regenerated incubating the spin column in 5 bed volumes of 0.2M NaOH in 95% ethanol for 1-2 hours at room temperature. The column was then washed with 5 bed volumes of 2M NaCl followed by 5 bed volumes of endotoxin free, ultra-pure water. The column was equilibrated with 5 bed volumes of sodium phosphate buffer (Na_2HPO_4 -1.42g in 10ml LPS free and autoclave, for NaH_2PO_4 add 1.2g in 10ml LPS free water and autoclave. The sample was added to the column and incubated overnight at room temperature and eluted the next day. The flow through was collected and samples were eluted in 1ml eppendorfs

2.22 Preparation of PBMCs

Dr. Ansar Pathan, Brunel Biosciences bled candidates willing to donate blood. This procedure was approved by the school's ethical committee. The blood was collected in a 50ml falcon tube containing 100 μ l of heparin to prevent clotting. Next, the blood was mixed with an equal amount of RPMI. Five 15ml falcon tubes were filled with 4ml of Ficoll and the blood was carefully transferred into each tube up to the top ensuring that the blood does not sink past the Ficoll layer. The falcon tubes were then spun at 2000 rpm for 15 minutes with the brake on the centrifuge off. Once the Ficoll had separated the blood components, the plasma, which was the top layer, was collected using a sterile Pasteur pipette into another falcon tube. The PBMCs, which appear as a white precipitate, were carefully sucked out using a Pasteur pipette and transferred into another 50 ml falcon tube. The next step involved filling the PBMCs to the top with RPMI

and spinning at 1500 for 10 minutes. The RPMI was discarded (which contained Ficoll) and cells were re-suspended in 50ml of fresh RPMI. The cells were then mixed by inverting the falcon tube and counted. The PBMCs were spun once again in the same conditions and re-suspended in 2ml of FCS. 2ml of 20% DMSO in RPMI was also added to the cells and the cells were aliquoted into 1ml cryogenic vials and frozen at -80 before transferring to liquid nitrogen.

2.23 Counting Cells (PBMCs)

10 μ l of cells were pipetted onto a section of a hemocytometre and a clear plastic slide placed on top which would allow the cells to flow by lamina flow. The haemocytometer was placed under the microscope and cells visible within 12 squares were counted with a cell counter. The total amount was multiplied by 10⁴ and spun at 1500 for 10 minutes, and resuspended in the equivalent amount of RPMI.

2.24 Preparation of Serum

AB Human Serum was used for the cellular assay. 50ml of the serum in a falcon tube was thawed at 37°C and heated in a water bath at 56°C for 30 minutes to inactivate complement components. Next, the serum was spun at 3000rpm for 30 minutes to spin down any debris. The serum was then filtered using a vacuum filter and aliquoted into 10ml samples and frozen at -20°C.

CHAPTER 3:

Examining the interaction between individual globular head modules of human C1q and its candidate receptor, gC1qR

3.1 Abstract

The gC1q domain of C1q holds a heterotrimeric configuration made up of three individual chains, ghA, ghB and ghC. The main receptor for this globular head region of C1q has been identified as gC1qR. There is very little known about the nature of interaction between the gC1q domain of C1q and gC1qR receptor. Here, we examined whether ghA, ghB, and ghC can interact independently with gC1qR. Although the binding site for C1q has been identified on gC1qR, we further sought to recognise the sites on the globular head modules that are complementary in this interaction via the expression and purification of single residue substitution mutants of each module. We have shown that ghA, ghB and ghC all bind independently to gC1qR. Consistent with previous data our results confirm that Arg¹⁶² of the ghA chain is central in facilitating the binding between gC1qR and C1q. In addition to this we have discovered that a single amino acid substitution of Arginine to Glutamate in residue 114 of the ghB module enhances the interaction and binds better than the wild-type ghB module. Mutational analysis revealed that the interaction is ionic and reliant on multiple contacts. In order to investigate a correlation between induced expression of C1q and gC1qR in adherent monocytes, we observed a concomitant rise in the levels of C1q and gC1qR within 48 hours of adherence. This was altered in a biphasic manner by exposure of monocytes to a PAMP i.e. LPS. We also examined if C1q mediated inhibition of PHA-stimulated PBMC proliferation is modulated by soluble gC1qR as well as recombinant globular modules. Our results suggest that interaction between the globular region and gC1qR is likely to be involved in the anti-lymphoproliferative activation of C1q.

3.2 Introduction

C1q is the first sub component of the classical complement pathway that links innate and adaptive immunity by virtue of recognising IgG and IgM on immune complexes (Reid et al, 1976). Structurally C1q is made up of 6A, 6B and 6C polypeptide chains. Each chain is followed by an N-terminal collagen region (CLR) and a C-terminal globular head domain (C terminal) of ghA, ghB or ghC (Kishore and Reid, 2000). The globular head modules come together to give rise to a heterotrimeric gC1q domain. C1q is able to bind to an array of self, non-self and altered self ligands (Reid, 1989; Reid et al, 2002; (Sim et al., 2007); Nayak et al, 2010; Kishore et al 2004) and perform multiple functions. (Nayak et al., 2010). The versatility of C1q is offered by the modular organisation of the individual globular heads A, B and C (Kishore et al., 2004); (Kojouharova et al., 2003); (Kishore et al., 2002); Kishore et al., 1998).

A number of cell surface receptors for C1q have been reported. C1qRp, also identified as CD93 (Steinberger et al., 2002) is a receptor for the CLR of C1q through its role in eliminating pathogens and immune complexes by enhancing C1q mediated phagocytosis by monocytes (Guan et al., 1994). CR1 is another CLR specific receptor for C1q which recognises IC opsonised by C3b and C4b (Medof et al., 1982). Although C1q also opsonises IC, research has shown that CR1 failed to remove IC in patients deficient for C3b and C4b which highlights that the interaction between C1q and this receptor is not essential for the clearance of IC. β 1 integrin is an additional collagenous binding complement receptor (Edelson et al., 2006) expressed on fibroblasts and endothelial cells (Zutter and Edelson, 2007). C1q has been demonstrated as a β 1 integrin adhesive ligand through its ability to bind this receptor on peripheral mononuclear cells allowing adhesion of these cells to complement containing immune complexes in a C1q and β 1 integrin manner (Zutter and Edelson 2007). Consequently this interaction is shown to stimulate cytokine secretion which suggests a role for this receptor in the innate immune response. CRT is an intracellularly expressed CLR binding receptor (Peerschke et al., 1994; Malhotra et al., 1994). Its involvement with C1q has been shown to play a role in the clearance of apoptotic and necrotic cells (Gregory and Devitt, 2004; Ogden et al., 2001). Here, C1q binds to PS on apoptotic cells via its gC1q domain and its CLR region is able to bind to CRT linked to CD91 on the monocytic cell surface (Basu et al.,

2001), leading to apoptotic cell phagocytosis. In addition to this, it has recently emerged that apoptotic cell clearance is also regulated by CRT being able to bind the globular region of C1q, with this interaction mediated by the CRT globular domain, (Kishore et al 1997a, 1997b; Paidassi et al., 2011).

A C1q receptor exclusive to the gC1q domain called gC1qR (33kDa) has been described. gC1qR is an highly acidic protein which is known to bind to the globular heads of C1q (Ghebrehiwet et al., 1994; Ghebrehiwet and Peerschke, 1998). Subsequently, gC1qR has emerged as a multi ligand binding and multi-functional protein, alongside binding to proteins involved in blood clotting such as thrombin and vitronectin (Lim et al., 1996). gC1qR activates the coagulation cascade through binding to HK on endothelial cells (Joseph et al 1996) which activates the vasoactive peptide BK, making the vessel wall more permeable, leading to an influx of tissue fluid (Herwald et al., 1996). This highlights a role for gC1qR in the control of inflammation. (See table 1.4 from chapter 1) Although the gC1q-gC1qR interaction was discovered a long time ago (Ghebrehiwet et al 1994), the complementary binding sites and the nature of interaction has not been established. The gC1q binding site on gC1qR is implicated to be located on residues 76-93 (Ghebrehiwet et al., 2002), The availability of the individual globular head fragments ghA, ghB and ghC as recombinant gC1q modules expressed in *E. coli* (Kishore et al., 2003) has given us the opportunity to reassess gC1q-gC1qR interaction and map the specific globular head gC1qR binds too. The structure function relationship within the gC1q domain is now better understood (Kishore et al 2003; Ghai et al 2007; Kishore et al 2005). It is now known that ghA, ghB and ghC are functionally independent modules. Together, as a heterotrimeric structure, this impact upon versatility and stabilisation for the range of ligands C1q can bind too. This is evident in the case of the HIV gp41 peptide 601-613 which preferentially binds to ghA (Kishore et al 2003) and the beta amyloid peptide specifically interacting with ghB, (Kishore et al 2003). With the modularity of gC1q explained, it is now to our understanding that each globular region functions differently in terms of ligand binding (Kishore et al 2003,

The crystal structure of gC1qR has been revealed as three monomers held together to form a trimer per gC1qR molecule (Jiang et al., 1999). Each monomer consists of seven anti-parallel β strands filled by an N-terminal and two C-terminal α helices. gC1qR has

different charge distribution, with one side of its 'donut' shaped structure exposed to be very negatively charged, the 'solution face' and the reverse being basic, the 'membrane face' (Ghebrehiwet et al 2002). The C1q binding site 76-94 is only exposed on the highly charged solution face, (Ghebrehiwet et al 2002). Given that the C1q binding site on gC1qR has been identified, we sought to identify the complementary residues on the gC1q domain that are involved in this interaction. Previous studies have highlighted Arg^{B114} and Arg^{B129} of the B chain to be central in the C1q-IgG interaction, (Kojouharova et al 2004) which is the first step in the activation of the classical pathway (Kishore and Reid, 2000). It has previously been shown that C1q binding to gC1qR on platelets and endothelial cells induces complement activation independent of IgG (Peerschke et al., 2006). Like C1q, gC1qR also has an anti-proliferative effect on B and T lymphocytes (Ghebrehiwet et al., 1990). In this chapter, the questions we have addressed are as follows:

1. What is the nature of complementary binding sites involved in the gC1q-gC1qR interaction?
2. Is there a concomitant relationship between expression of C1q and gC1qR in monocytes/macrophages under inflammatory conditions?
3. Do recombinant globular modules of C1q interfere with gC1q or C1q mediated anti-proliferative effect or stimulate PBMCs.

3.3 Materials and Methods

3.3.1 Proteins

C1q, Wild-Type ghA, ghB, ghC and their substitution mutants, gC1qR and its deletion mutants were produced as described in Materials and Methods

3.3.2 ELISA to examine interaction of ghA, ghB and ghC with gC1qR

ELISA was performed to examine the interaction between the C1q globular head modules and gC1qR. 1µg of gC1qR in carbonate - bicarbonate buffer was coated on microtitre wells overnight at 4°C. The microtitre wells were blocked with 2% BSA for 2 hours at 37°C. Next, the plate was washed with PBS + 0.05% Tween 20 three times. Different concentrations (2.5, 1.25, 0.625 and 0.312µg/well) of ghA, ghB or ghC (MBP as a control) were diluted in calcium buffer (Tris pH 8.8, CaCl₂ and 100mM NaCl) and added to wells. The plate was kept at 37°C for 1 hour and 4°C for 1 hour. Following further washes, bound protein was detected by anti-MBP mAb for 1 hour. After the addition of the secondary antibody rabbit anti-mouse IgG-HRP diluted in PBS (1:5000) and incubated for a hour at 37°C. The colour was developed using OPD and the plate was read at 450nm.

3.3.3 ELISA to examine binding of C1q to gC1qR

Microtitre wells were coated in duplicates with different concentrations of C1q (5, 2.5, 1.25 and 0.625µg/well) in carbonate- bicarbonate buffer, pH 9.6 overnight at 4°C. The microtitre wells were blocked with 2% v/w BSA in PBS for 1 hour at 37°C. Wells were then washed with PBS + 0.05% v/w Tween. 2.5µg of gC1qR was added to each well and incubated for an hour at 37°C. Following repeated washes, bound gC1qR was detected by anti-gC1qR pAb (1:1000). Protein A-HRP 1:1000 in PBS and incubated for a hour at 37°C. Colour was developed using OPD tablets

3.3.4 ELISA to examine binding of ghA module to gC1qR deletion mutants

Microtitre wells were coated overnight at 4°C in duplicates in carbonate-bicarbonate buffer with different concentrations (1, 0.5, 0.25µg/well) of each gC1qR deletion mutant (Δ144-148, Δ144-162, Δ174-180, Δ154-162Δ, 190-192, Δ196-202, Δ204-218, Δw233g,

Δ74-96.) The wells were blocked with 2% BSA in PBS for 2 hours at 37°C. Next, the wells were washed three times with PBS+0.05% Tween and 2.5μg/well of ghA in calcium buffer was added to each well and incubated for 1.5 hours at 37°C and 1.5 hours at 4°C. The wells were washed three more times with PBS + 0.05% Tween and incubated in 100μl of anti-MBP mAb (1:5000). The colour was developed after the use of rabbit-anti mouse IgG-HRP and OPD as described earlier.

3.3.5 Preparing Sheep Red Blood Cells (SRBCs)

5ml of sheep blood (Fisher Catalog No.: 50-177-000) was divided between two 50ml falcon tubes. Each tube was filled with 20ml of Dulbecco's PBS comprising of 0.9mM CaCl₂, 2.7mM KCL, 0.5mM MgCl₂H₂O, 138mM NaCl and 8.1mM Na₂HP0₄ and spun at 3000rpm for 10 minutes. The supernatant was carefully discarded without disrupting the pellet and the cells were washed three more times by re-suspending in Dulbecco's PBS (DPBS) using the same procedure. After the last wash, the cells in each tube were resuspended in 10ml of DPBS and the cell concentration was adjusted to 10⁹ per ml. This was carried out by taking 100μl of cells into a quartz cuvette and mixing with 1.4ml of dH₂O to cause complete haemolysis. The absorbance was read at a wavelength of OD₅₄₁ to 0.7 which represented 10⁹. If the OD was too high then the sample was further diluted with DPBS. One falcon tube of the 10⁹ cells were sensitised with 100μl of as Haemolysin (IgG and IgM) to yield the sensitised erythrocytes (EA). The falcon tubes were mixed through gentle inversions and incubated for 15 minutes each at 37°C ice. The cells were then spun at 3000rpm for 10 minutes and the supernatant was discarded. The pellet was re-suspended in DPBS and washed two more times and re-suspended in 50ml Dextrose Gelatin Veronal Buffer DGVB⁺⁺ comprising of 0.15mM CaCl₂, 141mM NaCl, 0.5Mm MgCl₂, 1.8mM Sodium Barbitol, 3.1mM Barbitone and 0.1% v/w gelatine. Next, the falcon tubes were centrifuged three times for 5 minutes and re-suspended in DGVB⁺⁺, with the first speed at 2000rpm, the second spin at 1000rpm, and 500rpm for the third wash. Cells were finally re-suspended in 10ml of DGVB⁺⁺. Both the EA and E cells (non-sensitised SRBCs) were stored at 4°C.

3.3.6 Testing EA and E cells for CH₅₀ value

To test the cells, 10 eppendorf tubes were labelled 1-10 and Normal Human Serum (NHS) was diluted to 1/10 in DGVB to give a final concentration of 200μl. 100μl of

DGVB⁺⁺ was added in tubes 2-10 and serial dilutions of the serum were performed by taking 100µl from tube 1 and mixing into each tube up to tube 10 with the final 100µl discarded. Another 10 eppendorf tubes were labelled 11-20 and the exact same procedure was carried. The samples were incubated at 37°C for 1 hour. Next, the EA cells and E cells were diluted to 10⁸/ml in DGVB⁺⁺ by adding 1ml of the cells to 9ml of dH₂O in a 50ml falcon tube. 100µl of E cells were added to 100µl of each serum dilution in tubes 1-10 and 100µl of EA cells to tubes 11-20. 100µl of EA cells with a 100µl of dH₂O was used as a positive control. The samples were incubated for a further 1 hour at 37°C. Next, the samples were spun at 5000rpm for 5 minutes and 180µl of the supernatant was transferred onto a microtitre plate and read at an OD of 405nm.

3.3.7 Western Blotting

To verify the immunoreactivity of the recombinant gC1qR expressed and purified, western blotting was performed. 10µg of recombinant gC1qR was run on an 12% v/w SDS-PAGE gel and transferred for 1 hour onto PDVF membrane. The membrane was blocked for 1 hour at room temperature with 5% non-fat milk in PBS, followed by pAb anti-gC1qR (1:1000) incubated at room temperature overnight. Following this the membrane was washed 3 times in PBS-Tween 0.02% and probed with PA-HRP in PBS, for 1 hour at room temperature (1:1000). Colour was developed using DAB.

3.3.8 Far Western Blot to show gC1qR binding to ghA, ghB and ghC.

10µg of ghA, ghB, ghC and MBP was run on a gel and transferred onto a PDVF membrane for 1 hour at 320m. The membrane was then blocked for 2 hours with 5% non-milk fat powder in PBS at room temperature. 15µg of gC1qR in PBS was added to the membrane and incubated overnight at room temperature. The next morning, the membrane was washed 3 times for 20 minutes each with 0.02% PBS-Tween 20. Anti-gC1qR pAb (1:1000) was added in 2% non-fat milk in PBS and the membrane was incubated for 2 hours at 37°C. Next, the membrane was washed as mentioned above and Protein A-HRP (1:1000) was diluted in 2% non-fat milk PBS and flooded on the membrane for 1 hour at room temperature. Once again the membrane was washed and the bands were developed using DAB tablets (Sigma, Cat no: D7304) dissolved in 15ml of H₂O.

3.3.9 Far Western Blot to show ghA, ghB and ghC binding to gC1qR

15µg of gC1qR was loaded on a 12% SDS-PAGE gel in three wells and run at 120v. The gel was cut into three sections and transferred onto PDV membrane for 1 hour at 320ma. The membrane was also cut into three strips and blocked with 5% non-fat milk in PBS for 2 hours at room temperature. 10µg of ghA, ghB and ghC in Calcium buffer were added to each strip and incubated overnight at room temperature. The membranes were then washed 3 times in PBS-Tween for 20 minutes each and 10µl of anti-MBP mAb in 2% non-fat milk was added to each membrane and incubated for 2 hours at 37°C. Following washing 3 times in PBS-Tween for 20 minutes each, 10µl of rabbit anti-mouse IgG-HRP in 2% non-fat milk was added and incubated for 1 hour at room temperature. The bands were developed using DAB tablets dissolved in water.

3.3.10 Assay for the solid and solution phase complement activation by recombinant gC1qR

To see whether recombinant gC1qR produced using a new expression system was able to activate complement in solution as well as solid phase, haemolytic assays were performed. Different concentrations of gC1qR (10, 5, 2.5, 1.25, 0.625µg) were incubated at 37°C for 1 hour with NHS in DGVB⁺⁺ (at a dilution of 1:40) to give a total volume of 100µl. Then, 100µl of EA cells (1×10^8) were then added to each sample and incubated for 1 hour. Samples were spun down for 5 minutes at 5000rpm and 100µl of the supernatant was collected and read at 520nm. dH₂O and EA cells were used for 100% lysis. For solid phase studies, gC1qR was bound to microtitre wells at the same concentrations overnight at 4°C. NHS in DGVB⁺⁺ was added using the same procedure mentioned above and the assay was developed in the same manner

3.3.11 Complement assays using gC1qR deletion mutants

Microtitre wells were coated with 4µg/ml of each gC1qR deletion mutant, Δ144-148, Δ144-162, Δ174-180, Δ154-162Δ, 190-192, Δ196-202, Δ204-218, Δw233g, Δ74-96, WT gC1qR and heat-aggregated IgG in carbonate-bicarbonate buffer, p.H 9.8 and incubated at 37°C for 2 hours. Contents were discarded and the wells were blocked for 1 hour with 2% BSA. Next, wells were washed 3 times with DGVB⁺⁺ and 100µl of 1/10 dilution of NHS in DGVB⁺⁺ was added to each well and incubated for 1 hour at 37°C. The wells were repeatedly washed three times in DGVB⁺⁺ and biotinylated anti-C4d (1:2000) diluted in

DGVB⁺⁺ was added to the wells and incubated for a hour at 37°. The secondary antibody used was Neutravidin conjugated to Alkaline Phosphatase (AP) diluted in DGVB⁺⁺, was incubated for an hour at 37°C. Following repeated washing with DGVB⁺⁺, bound C4d was detected using Para-nitrophenylphosphate (PNPP) substrate.

3.3.12 Expression analysis of C1q and gC1qR following monocyte adherence and LPS challenge

3.3.12.1 Preparation of PBMCs

Whole blood (50ml) was taken from healthy consenting human subjects and 2 units/ml of heparin (Wockhardt) added to prevent clotting. Blood was then diluted with an equal volume of RPMI 1640 (Sigma-Aldrich). To isolate monocytes, blood/RPMI was separated on a Ficol column (Ficol-Plaque Plus, GE healthcare) by centrifugation at 2000 rpm for 16 minutes at room temperature. Following the removal of the top serum layer, subsequent peripheral blood mononuclear cell (PBMC) interphase layer was carefully removed. An equal volume of RPMI 1640 was then added and the cells pelleted by centrifugation at 1500 rpm for 10mins at room temperature. Cells were then resuspended in 50mls of RPMI and the cell concentration determined using a hemocytometer. Approximately 7×10^7 cells were obtained in total.

3.3.12.2 RNA extraction and qPCR,

Approximately 5×10^6 cells were added to each culture plate in 5 ml of RPMI 1640, containing 10% Fetal Calf Serum (FCS), 100µg/ml penicillin-streptomycin and 2mM L-glutamine. Cells were then incubated at 37°C with 5% CO₂ atmosphere and left to adhere. Cells were then harvested at the following time points of incubation for adherence: 2 hrs, 24hrs, 48hrs, 72hrs, 5 days and 7 days. Identical cultures at the above time-points were also setup with the addition of 20ng/µl of LPS (*Salmonella typhimurium*, Sigma-Aldrich). Adherent cells with or without LPS from each timepoint were harvested by removing the media from the plate and incubating cells in RPMI 1640, containing 0.025% trypsin/ 0.01% EDTA for 5 mins at 37°C. Cells were removed using a cell scraper and an equal volume of RPMI 1640, containing 10% FCS added to the harvested cells. Cells counts were then determined on a haemocytometer. Cells were

pelleted by centrifugation at 1500 rpm for 10 mins at room temperature and the supernatant removed, and cell pellets stored at -80°C until RNA extraction procedure

Total RNA was extracted from frozen cell pellets using the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich). Samples were then treated with DNase I (Sigma-Aldrich) to remove any contaminating DNA. Samples were then heated at 70°C for 10 min to inactivate both the DNase I and the RNase, and subsequently chilled on ice. The amount of total RNA was measured by determining the absorbance at 260nm using the NanoDrop 2000/2000c (Thermo-Fisher Scientific) and the purity of the RNA determined using the ratio of absorbance at 260nm and 280 nm. Complementary DNA (cDNA) was synthesized using High Capacity RNA to cDNA Kit (Applied Biosystems) from a quantity of 2µg of total RNA extract.

Primer sequences were designed and analyzed for specificity using the nucleotide Basic Local Alignment Search Tool and Primer-BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The following primers were used: For 18S rRNA gene (endogenous control): *18S* forward (5'-ATGGCCGTTCTTAGTTGGTG-3'), *18S* reverse (5'CGCTGAGCCAGTCAGTGTA-3'), for C1q gene: *qC1qC* forward (5'CAAAGGGCAGAAGGGAGAAC-3'), *qC1qC* reverse (5'ATCTGATCAGGCTGTTGGGT-3') and for gC1qR gene: *gC1qR* forward (5'AACAACAGCATCCCACCAAC-3'), *gC1qR* reverse (5'AGATGTCACTCTCAGCCTCG-3').

PCR was performed on all cDNA samples to assess the quality of the cDNA. The qPCR assays were performed for the expression C1qR and gC1qR. The qPCR reaction consisted of 5 µl Power SYBR Green MasterMix (Applied Biosystems), 75 nM of forward and reverse primer, 500 ng template cDNA in a 10 µl final reaction volume. PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The initial steps were 2 minutes incubation at 50°C followed by 10 minutes incubation at 95°C, the template was then amplified for 40 cycles under these conditions: 15 seconds incubation at 95°C and 1 minute incubation at 60°C. Samples were normalized using the expression of human 18S rRNA. Data was analyzed using the RQ Manager Version 1.2.1 (Applied Biosystems). Ct (cycle threshold) values for each target gene were calculated and the relative expression of each cytokine target gene was calculated using the Relative Quantification (RQ) value, using the formula: $RQ = 2^{-\Delta\Delta Ct}$ for each cytokine

target gene, and comparing relative expression with that of the 18S rRNA constitutive gene product. Assays were carried out twice in triplicate.

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software). An unpaired 2-side t-test was used to compare the means of the expressed targets of the timepoints analyzed, using the 2h timepoint as calibrator. P values were computed, graphs compiled and analysed.

3.3.13 Cell Proliferation Assay

3.3.13.1 Titrating PHA (Phytohaemagglutinin)

To work out the optimum concentration of PHA required to stimulate PBMCs, pha was titrated with cells. Three peoples PBMCs vials were removed from liquid nitrogen and thawed at 37°C. Three falcon tubes were filled to 10ml with RPMI containing Fetal Calf Serum and each vial of cells were added to a tube and spun at 1500 for 10 minutes. The cells were then resuspended in 5ml RPMI and counted. Next the cells were spun at 1500 for 10mins and resuspended in the correct volume of prepared medium, consisting of RPMI, L-glutamine, Pen strap, sodium pyruvate and AV serum to give a concentration of 0.5 million cells per ml. 100,000 cells of each persons PBMCs were added per well into sterile U bottom microtitre culture plates. Following this, various concentrations of PHA, (1, 0.5 and 10µg/ml) were added to the cells and placed in a CO₂ incubator to allow proliferation to take place. Three days later the plate was pulsed, harvested and counted as described.

3.3.13.2 Cell proliferation assay

The PBMCs of three different subjects were used to carry out the proliferation assay. PBMCs frozen in liquid nitrogen were thawed at 37°C until a slight bit of ice remained. The cells were transferred into 3 falcon tubes containing 10ml of RPMI with FCS. The samples were spun at 1500rpm for 10 minutes and the cells were resuspended in 10ml of RPMI. The cells were counted using the procedure mentioned in the materials and methods section and spun at 1500rpm for 10 minutes. The cell volumes were resuspended accordingly with RPMI medium (stock solution: 87ml RPMI, 1ml L-Glutamine, 1ml Pennicilin-Streptomycin, 1ml Sodium Pyruvate and 10ml filtered AB serum). Half of the cells were treated with 1µg/ml of PHA and 700µl of this solution was

added to each eppendorf tube. 10µg of the antigens ghA, ghB, ghC, gC1qR, MBP or C1q were also added to each sample of cells and mixed by vortexing. The same procedure mentioned above was carried out with the PBMCs unstimulated with PHA to be used as negative controls. The experiment was carried out in triplicates; therefore 200µl of each sample was pipetted three times into a sterile, round 'U' bottom microtitre well plate. The plate was then placed in a 37°C CO₂ incubator and pulsed with thymidine on day 3. The cells were harvested on day 4 and counted.

3.4 Results

Expression and purification of C1q globular head substitution mutants

Table 3.1: List of globular head mutants expressed and purified

Globular head region	Mutant
ghA	R162A
	R162E
ghB	T175L
	R114A
	R114Q
	R129A
	R129E
	R163A
	R163E
	L136G
	H117D
ghC	R156Q
	R156E
	H101A
	L170E

ghA, ghB and ghC single residue substitution mutants were expressed and purified to determine important residues in the C1q globular head that play a role in ligand binding.

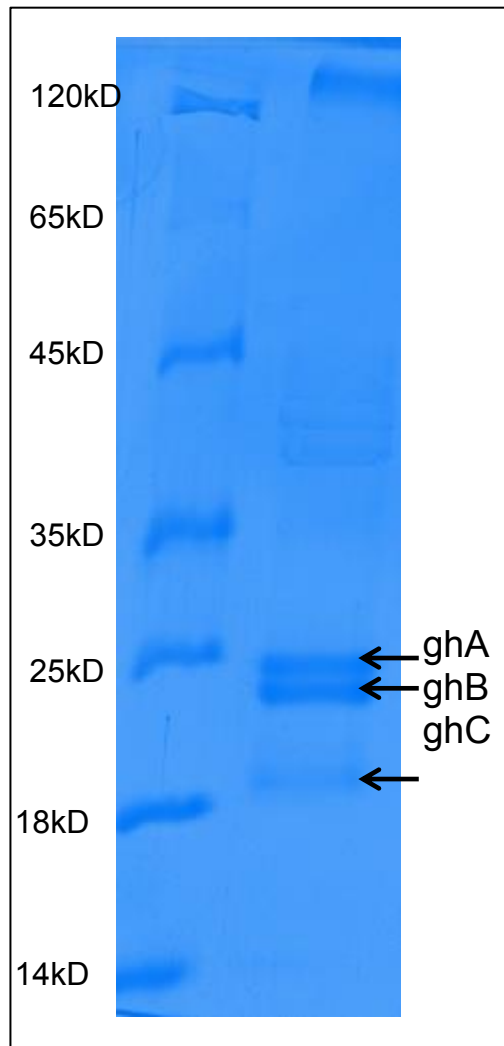


Figure: 3.1: 7.5 % w/v SDS-PAGE of purified human C1q under reducing conditions. The three chains of C1q can easily be seen. The higher bands represent dimers and multimers of the C1q C chain.

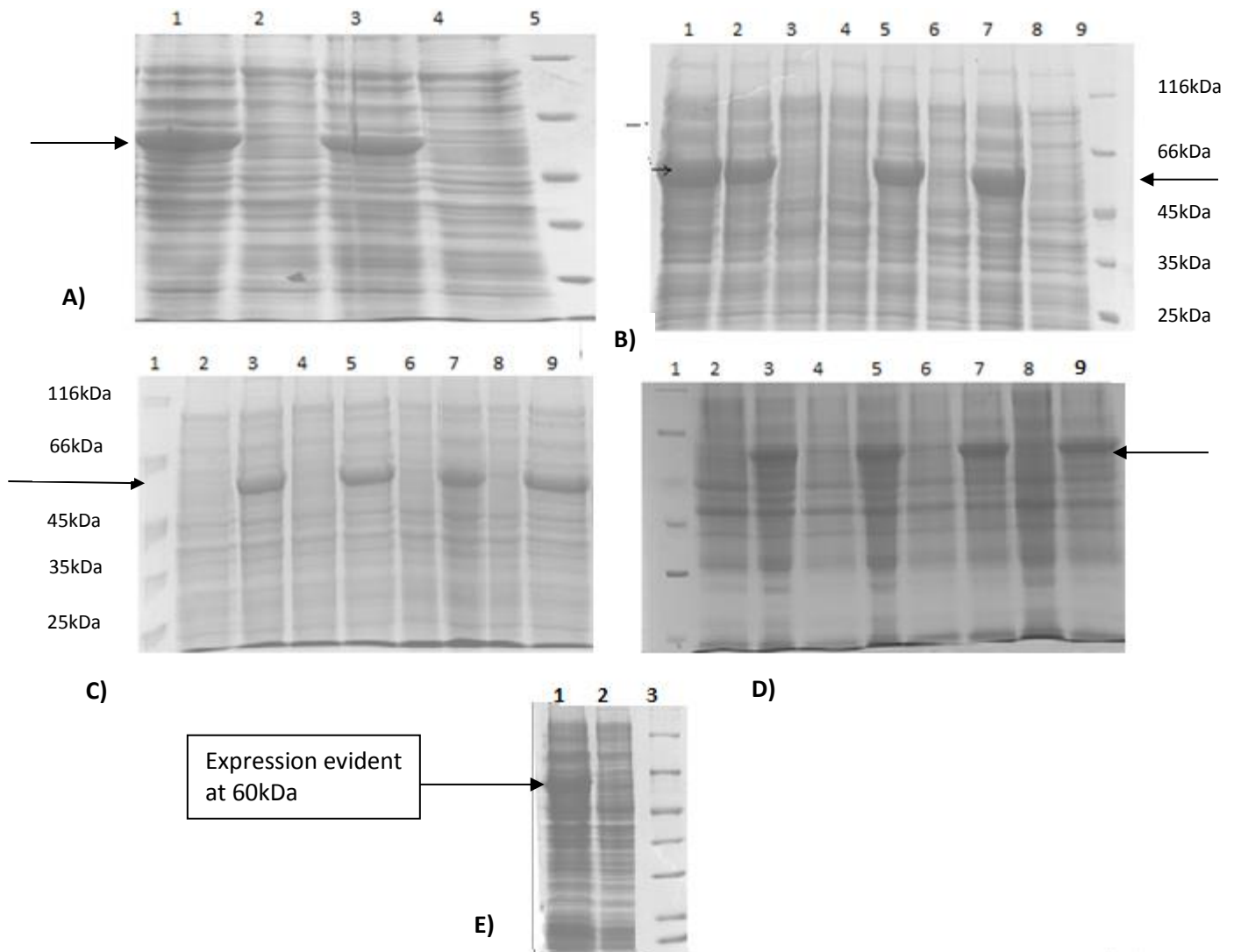


Figure 3.2: (12% under reducing conditions). SDS-PAGE showing expression of the MBP-linked globular head mutant fusion proteins ghA-R162A, ghA-R162E, ghB-R163E, ghB-R163A, ghB-R114A, ghB-R114Q, ghB-R114Q, ghB-R129A, ghB-R129E, ghB-T175L, ghB-L136G, ghB-H117D. *E. coli* BL21 strain individually transformed with plasmids containing mutant forms of C1q globular head modules was induced with IPTG for 3 hours and a cell pellet of 100µl of bacterial culture was boiled under reduced conditions and loaded in each lane. Bacterial cells, grown in parallel but not induced with IPTG were used as an uninduced control. **A)** Lane 1: induced sample of ghA-R162A, Lane 2: Uninduced sample of ghA-R162A. Lane 3: Induced sample of ghA-R162E. Lane 4: uninduced sample of ghA-R162E, Lane 5: Molecular marker. **B)** Lane 1: Induced sample of ghB-R163E, Lane 2: Induced sample of ghB-R163A, Lane 3: Uninduced ghB-R163A, Lane 4: Uninduced ghB-R163E, Lane 5: Induced ghB-R129A, Lane 6: Uninduced ghB-R129A, Lane 7: Induced ghB-R129E, Lane 8: Uninduced ghB-R129E, Lane 9: **C)** Lane 1: Molecular marker, Lane 2: Uninduced ghB-R114Q, Lane 3: Induced ghB-R114Q, Lane 4: Uninduced ghB-R114A, Lane 5: Induced ghB-R114A, Lane 6: Uninduced ghB-H117D, Lane 7: Induced ghB-H117D, Lane 8: Uninduced ghB-T175L, Lane 9: Induced ghB-T175L. **D)** Lane 1: Molecular marker, Lane 2: Uninduced ghB-L136G, Lane 3: Induced ghB-L136G, Lane 4:

Uninduced ghC-R156Q, Lane 5: Induced ghC-R156E, Lane 6: Uninduced ghC-R156E, Lane 7: Induced ghC-R156E, Lane 8: Uninduced ghC-H101A, Lane 9: Induced ghC-H101A, **E)** Lane 1: Induced ghC-L170E, Lane 2: Uninduced ghC-L170E, Lane 3: Molecular marker. Expression for all samples shown under the 66kda marker

3.4.1 Amylose Resin Purification

Following lysis and sonication, the supernatant of each protein was subjected to purification on an amylose resin column. Since the globular heads were expressed as fusion proteins to MBP, an amylose resin column was used, which would enable MBP to bind to the column and all other *E.coli* proteins would flow through. The bound MBP fused proteins were eluted by 10mM Maltose. The elutions were run on a 10% SDS-PAGE to assess for purity. As shown in figure 3.3, the fusion proteins had significant *E.coli* protein contamination with the exception of ghA-R162A, ghA R162E and ghB-R162A, which appeared relatively clean. Therefore the fusion proteins were subjected to ion exchange for further purity (Figure 3.5).

3.4.2 Ion Exchange of globular head substitution mutants

Ion exchange uses a positively charged solid support to attract the charge of a negatively charged protein. In this case we used an anion column made up of DEAE Sepharose. Once the protein is bound to the column, Sodium Chloride is used to elute the protein. In ion exchange, chloride is the counter ion as chloride is exchanged for the protein on the matrix which allows the protein to be displaced and elute. A gradient was performed where different NaCl concentrations ranging from 0.1-0.6M was passed through the column and eluted protein was run on a SDS-PAGE gel. Following dialysis against 100mM NaCl the mutants ghB-R163E, ghB-R114A, ghB-R114Q, ghB- R129A, ghB-R129E, ghB- H117D, ghB- L136G, ghB-T175L, ghC-R156E, ghC-H101A and ghC-L170E were passed through DEAE sepharose and eluted at a known concentration of 0.4M (Kishore et al 2004). Samples were collected in 1ml fractions and run on a 10% SDS-PAGE gel to assess for purity.

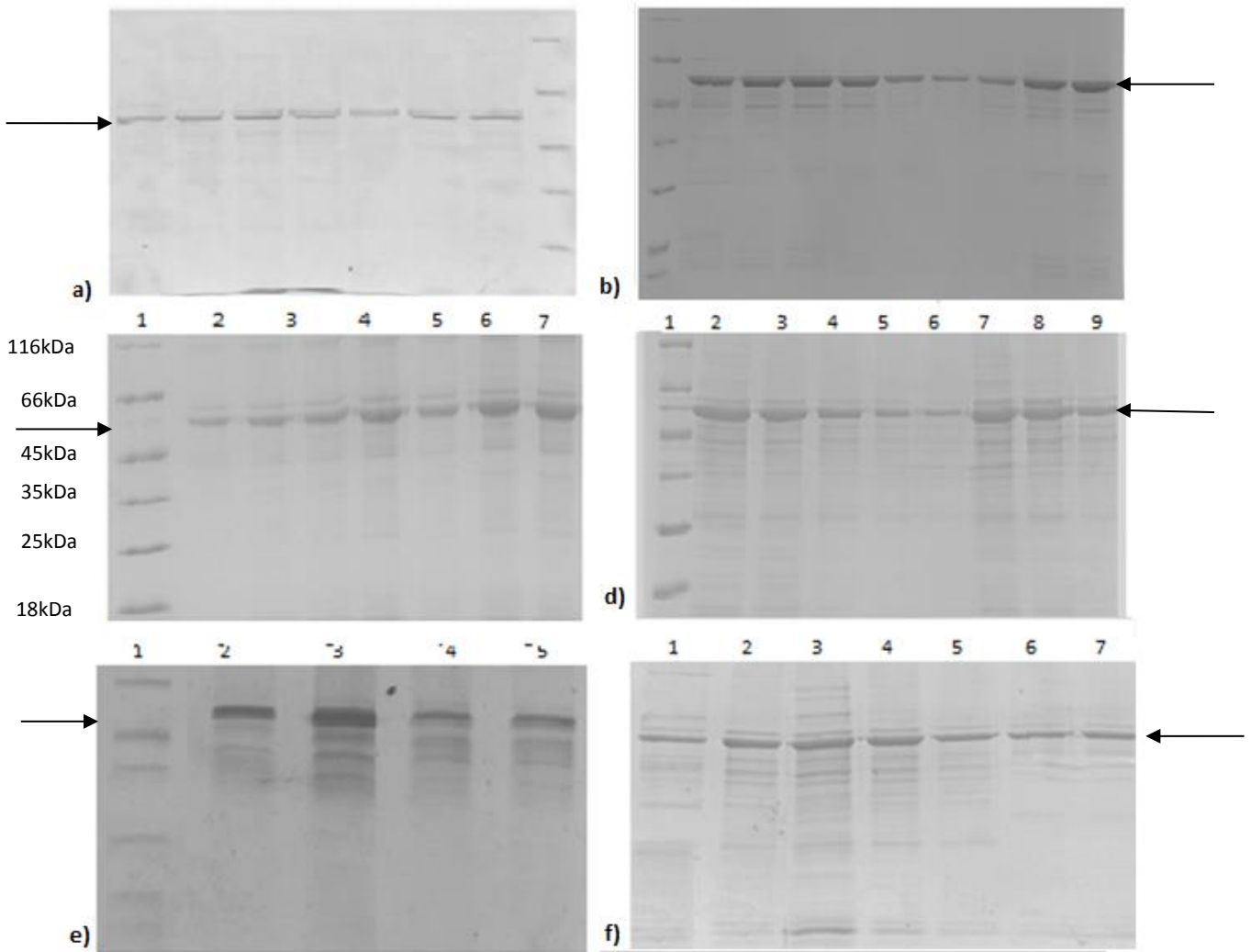


Figure 3.3: Purified membrane fraction of MBP-fused ghA-R162A, MBP-fused ghA-R162E, MBP-fused ghB-R163A, MBP-fused ghB-R163E , MBP-fused ghB-R114A, MBP-fused ghB-R114Q, MBP fused ghB-R129A, MBP fused ghB-R129E, after flowing through Amylose resin column. Following sonication, samples were run through an Amylose Resin column and eluted with 10mM Maltose. 2ml membrane fractions were collected and run on a 10% gel. **a)** Lane 1 – 7: Purified ghA-R162A,; Lane 8: Molecular marker, **b)** Lane 1: Molecular marker, Lane 2-5: Purified ghA-R162E, lane 6-10: Purified ghB R163A. **c)** Lane 1: Molecular marker, Lane 2-5: Purified ghB- R163E, **d)** Lane 1: Molecular marker, Lane 2-6: Purified ghB-R114A, Lane 7-9: Purified ghB-R114Q. **e)** Lane 1: Molecular marker, Lane 2-5: Purified ghB-R129A. **f)** Lane1-7: Purified ghB-R129E. Expression is evident corresponding to the 66kda marker.

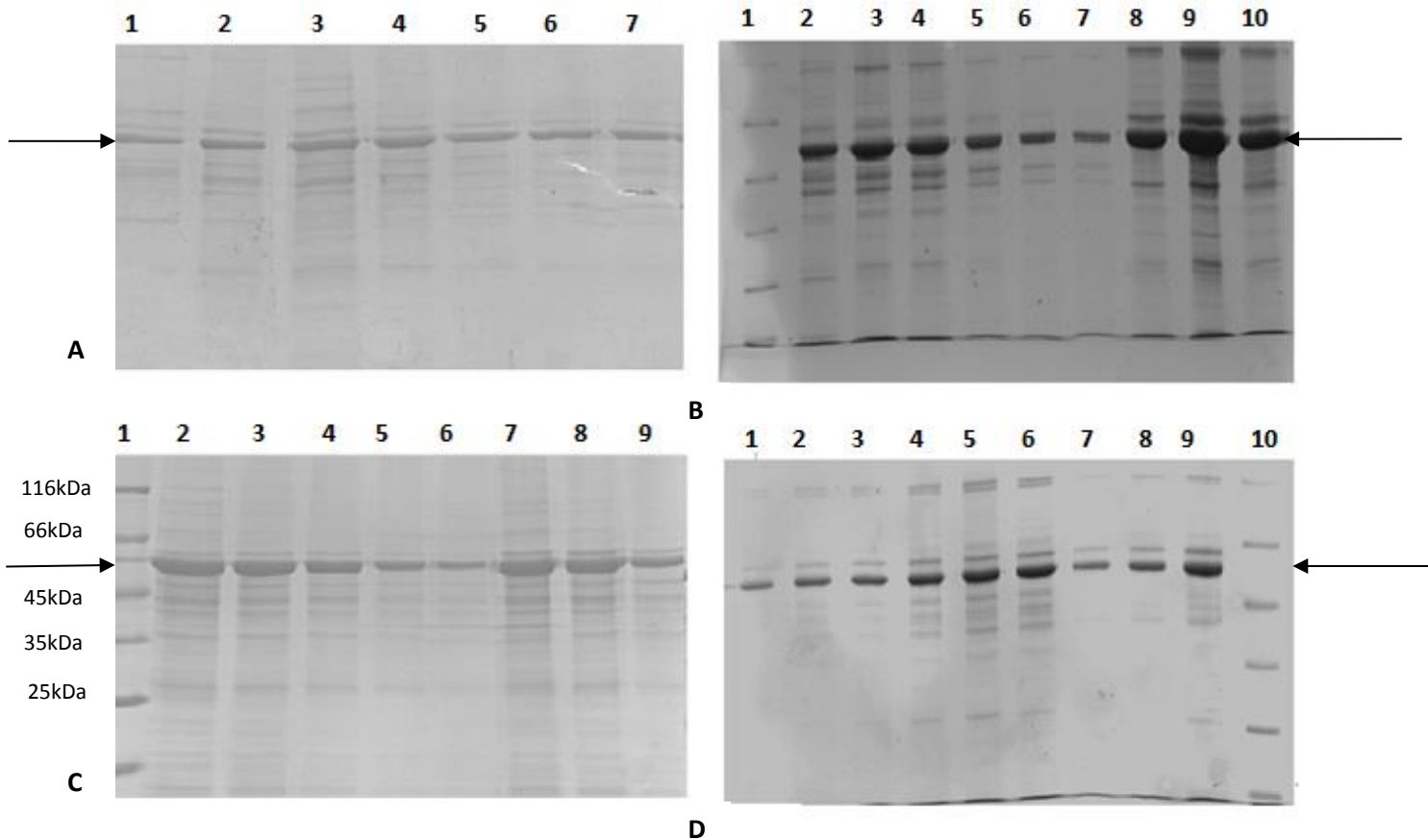


Figure 3.4: Purified membrane fraction of MBP-fused ghB-H117D, MBP-fused ghB-L136G, MBP-fused ghB-T175L, MBP-fused ghC-R156E , MBP-fused ghC-H101A, MBP-fused ghC-L170E, after flowing through Amylose resin column. Following sonication, samples were run through an Amylose Resin column and eluted with 10mM Maltose. 2ml membrane fractions were collected and run on a 10% gel. **a)** Lane 1 – 6: Purified ghB-H117D, **b)** Lane 1: Molecular marker, Lane 2-7: Purified ghB-L136G, lane 8-10: Purified ghB-T175L. **c)** Lane 1: Molecular marker, Lane 2-6: Purified ghC- R156E, lane 8-10: Purified ghC-H101A **d)** Lane 1-9: Purified ghC-L170E, Lane 10: Molecular marker, Expression is evident corresponding to the 66kda marker.

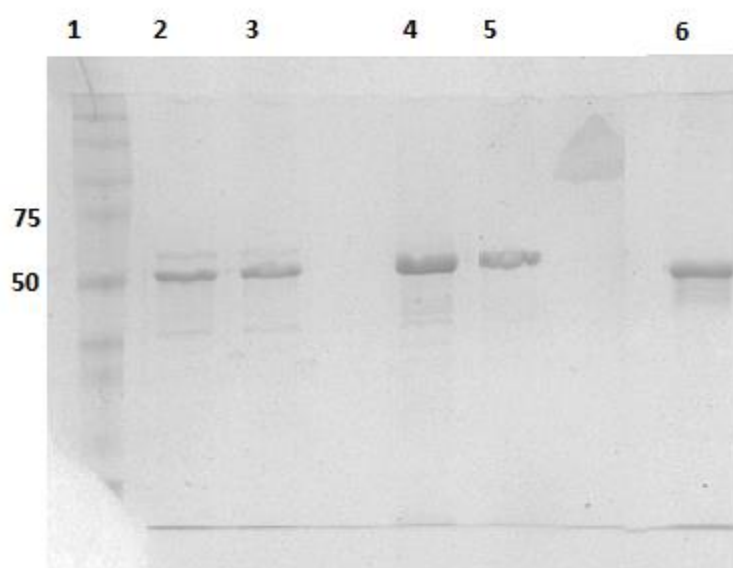
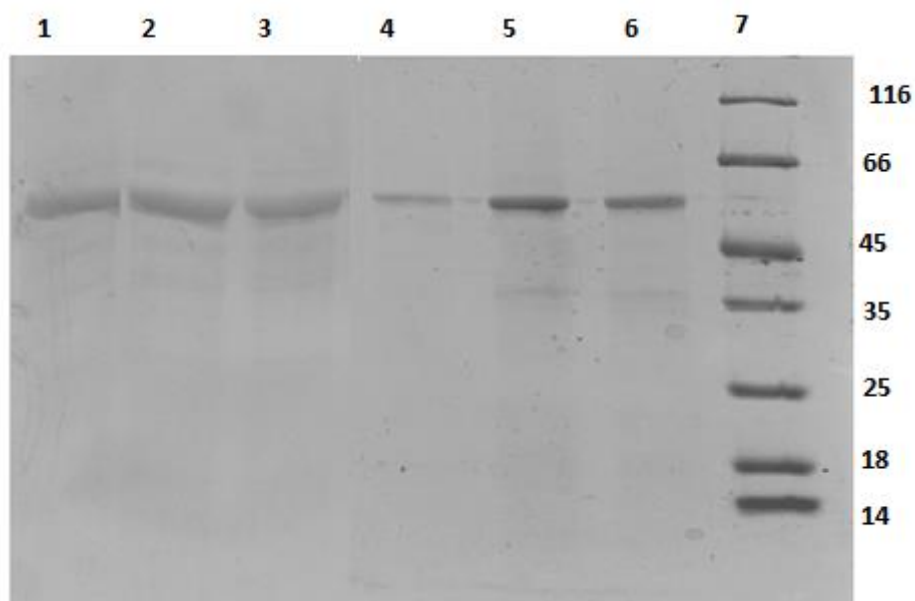


Figure 3.5: Purified membrane fraction of globular head mutants after ion exchange. Following purification on amylose resin column, samples were dialysed against 20mM Tris and 100mM NaCl and loaded on a DEAE sepharose column. Fractions were eluted at 0.6M NaCl and run on a 10% v/w SDS gel. **A)** Lane 1: Purified ghB-R163E, Lane 2: Purified ghB-R114A, Lane 3: Purified ghB-R114Q, Lane 4: Purified ghB-R129A, Lane 5: Purified ghB-R129E, Lane 6: Purified ghB-H117D, Lane 7: Molecular marker. **B)** Lane 1: Molecular marker, Lane 2: Purified ghB-L136G, Lane 3: Purified ghB-T175L, Lane 4: Purified ghC-R156E, lane 5: Purified ghC-H101A, lane 6: Purified ghC-L170E

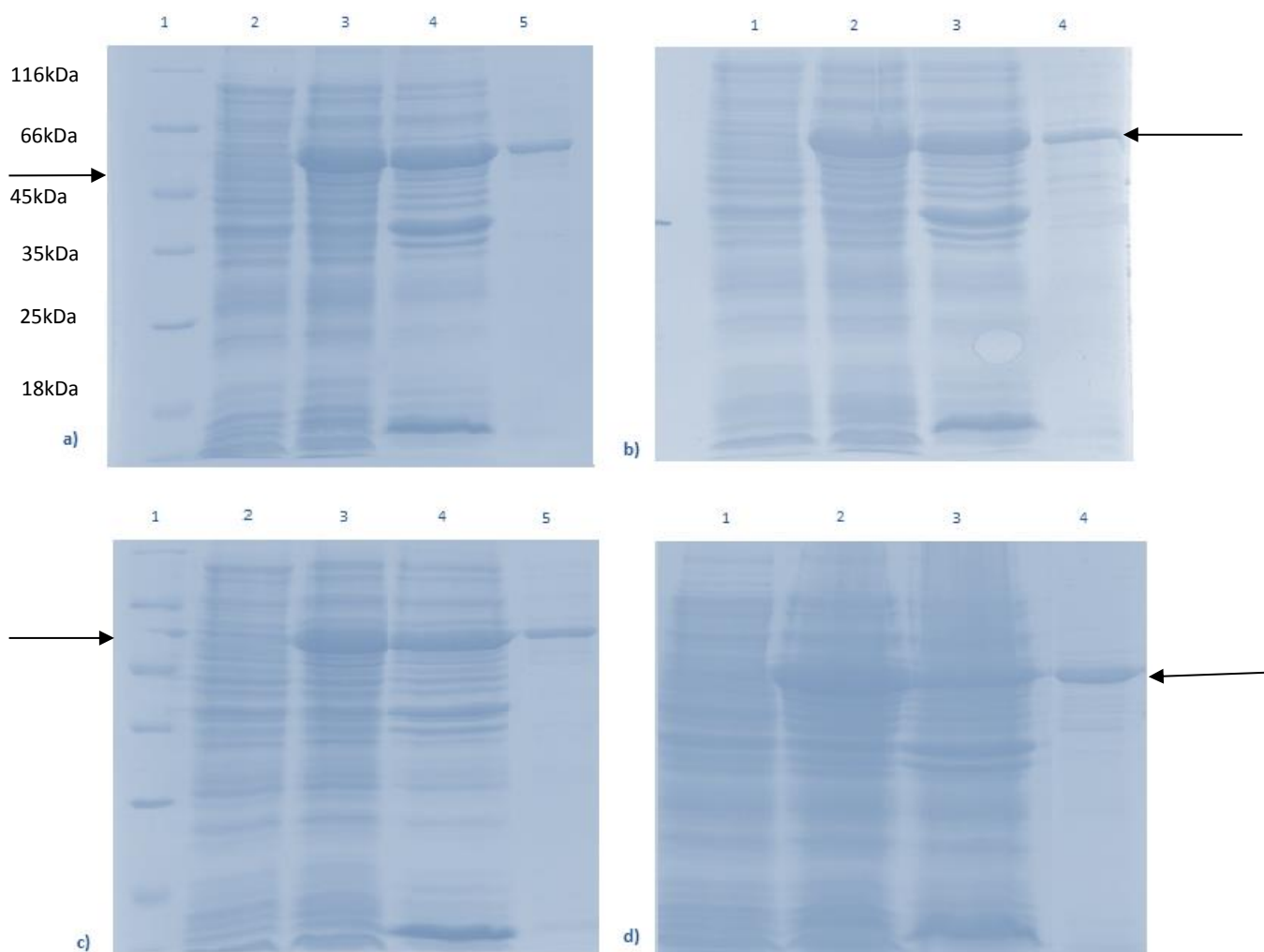


Figure 3.6: Expression and purification of MBP-fused globular head domains ghA-R162A, ghA-R162E, ghB-R114A, ghB-R114Q, a) Lane 1: Molecular marker, Lane 2: Uninduced ghA-R162A, Lane 3: Induced R162A, Lane 4: Supernatant of R162A following lysis and sonication, Lane 5: Purified R162A following Amylose resin chromatography, **b)** Lane 1: Uninduced ghA-R162E, Lane 2: Induced R162E, Lane 3: Supernatant of R162E following lysis and sonication, Lane 4: Purified R162E fraction following Amylose resin chromatography, **c)** Lane 1: Molecular marker, Lane 2: Uninduced ghB-R114A, Lane 3: Induced R114A, following amylose exchange chromatography, **d)** Lane 1: Uninduced ghB-R114Q, Lane 2: Induced R114Q, Lane 3: Supernatant of R114Q after lysis and sonication Lane 4: Purified R114Q following amylose resin chromatography.

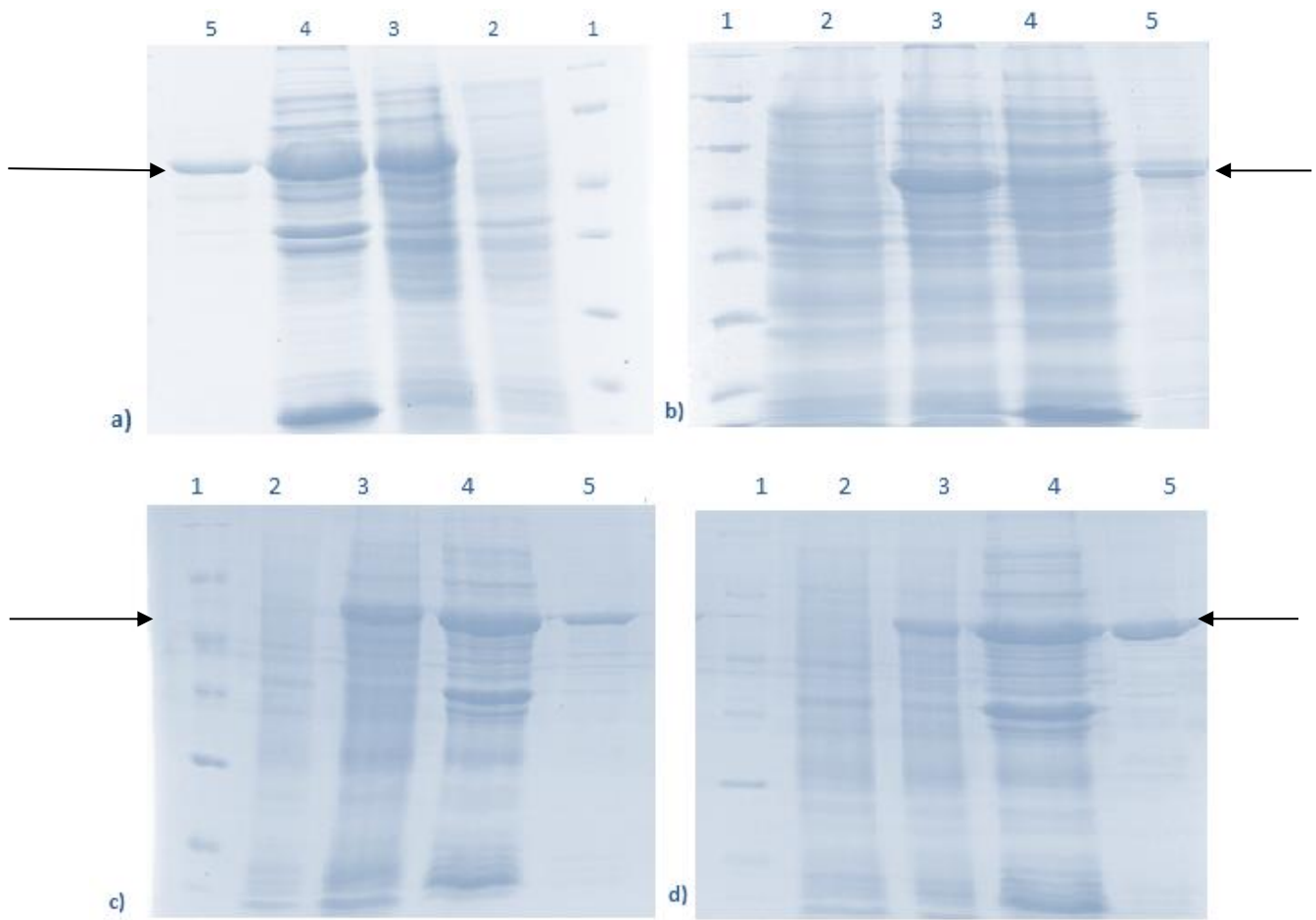


Figure 3.7: Expression and purification of MBP-fused globular head domains ghB-R163A, ghB-R163E, ghB-R129A, ghB-R129E, a) Lane 1: Molecular marker, Lane 2: Uninduced ghB-R163A, Lane 3: Induced R163A, Lane 4: Supernatant of R163A following lysis and sonication, Lane 5: Purified R163A following Amylose resin chromatography, **b)** Lane 1: Molecular marker, , Lane 2: Uninduced ghB-R163E, Lane 3: Induced R163E, Lane 4: Supernatant of R163E following lysis and sonication, Lane 5: Purified R163E fraction following Amylose resin chromatography, **c)** Lane 1: Molecular marker, Lane 2: Uninduced ghB-R129A , Lane 3: Induced R129A, Lane 4: Supernatant of R129A following lysis and sonication, Lane 5: Purified R129A following amylose exchange chromatography, **d)** Lane 1: Molecular marker, Lane 2: Uninduced ghB-R129E, Lane 3: Induced R129E, Lane 4: Supernatant of R129E after lysis and sonication Lane 5: Purified R129E following amylose resin chromatography.

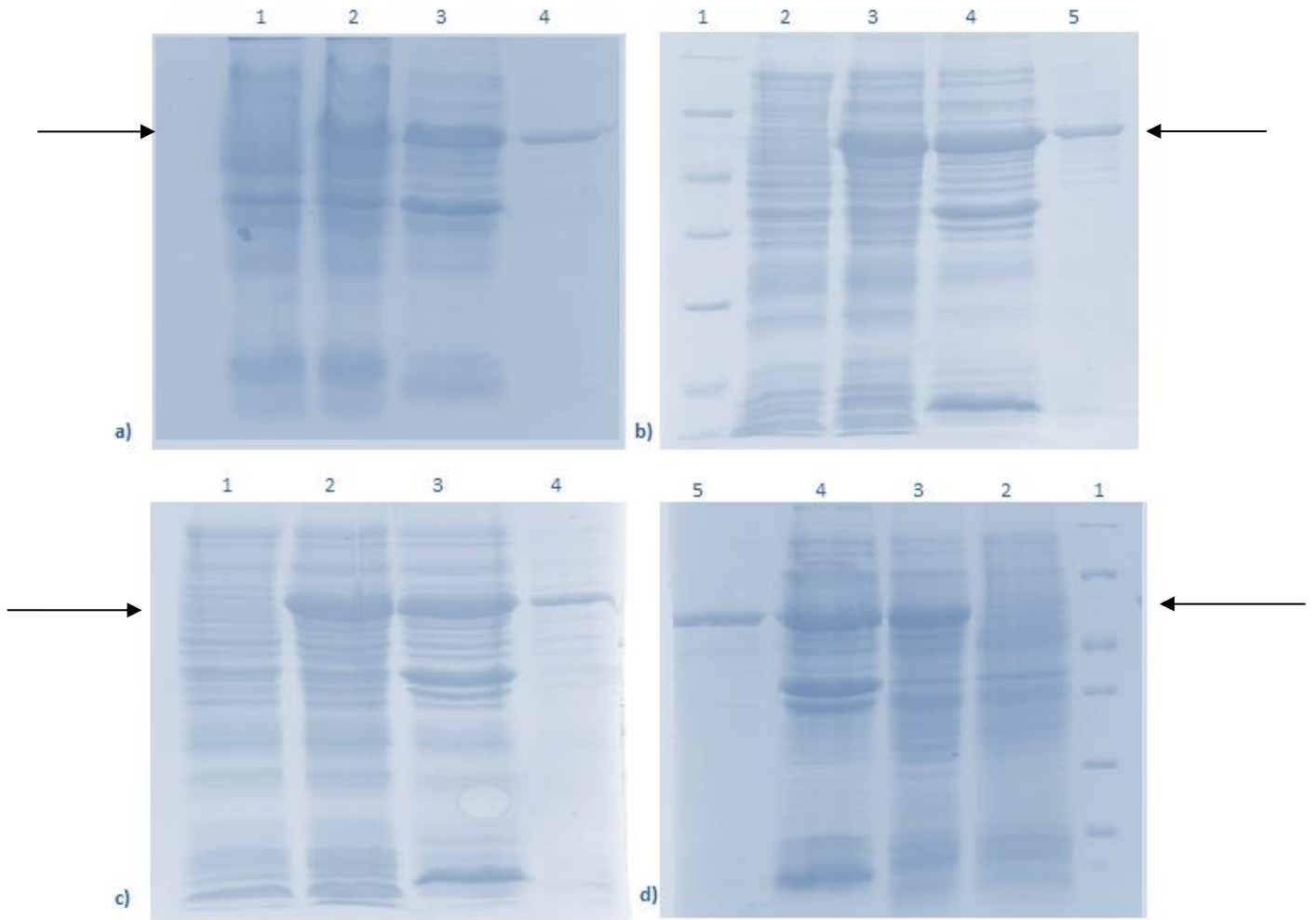


Figure 3.8: Expression and purification of MBP-fused globular head domains ghB-H117D, ghB-T175L, ghB-L136G, ghC-R156Q, a) Lane 1: Uninduced ghB-H117D Lane 2: Induced H117D, Lane 3: Supernatant of H117D following lysis and sonication Lane 4: Purified H117D following Amylose resin chromatography **b)** Lane 1: Molecular marker, Lane 2: Uninduced ghB-T175L, Lane 3: Induced T175L, Lane 4: Supernatant of T175L following lysis and sonication, Lane 5: Purified T175L fraction following Amylose resin chromatography, **c)** Lane 1: Uninduced ghB-L136G, Lane 2: Induced L136G Lane 3: Supernatant of L136G following lysis and sonication, Lane 4: Purified L136G following amylose exchange chromatography, **d)** Lane 1: Molecular marker, Lane 2: Uninduced ghC-R156Q, Lane 3: Induced R156Q, Lane 4: Supernatant of R156Q after lysis and sonication Lane 5: Purified R156Q following amylose resin chromatography.

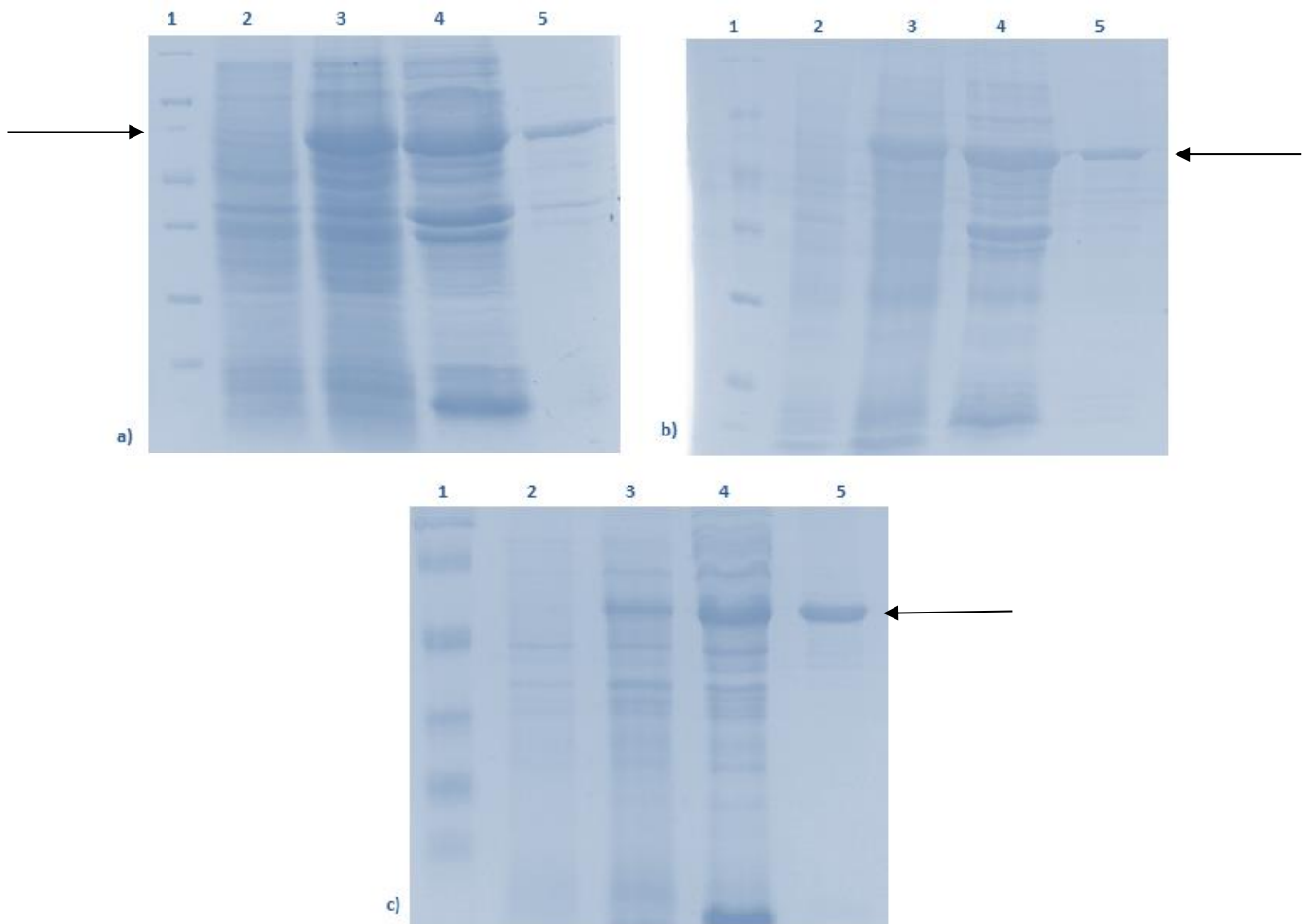


Figure 3.9: Expression and purification of MBP-fused globular head domains ghC-R156E, ghC-L170E, ghC-H101A, a) Lane 1: Molecular marker, Lane 2: Uninduced ghC-R156E, Lane 3: Induced R156E, Lane 4: Supernatant of R156E following lysis and sonication Lane 5: Purified R156E following Amylose resin chromatography b) Lane 1: Molecular marker, Lane 2: Uninduced ghC-L170E, Lane 3: Induced L170E, Lane 4: Supernatant of L170E following lysis and sonication, Lane 5: Purified L170E fraction following Amylose resin chromatography, c) Lane 1: Molecular marker, Lane 2: Uninduced ghC-H101A, Lane 3: Induced H101A, Lane 4: Supernatant of H101A following lysis and sonication, Lane 5: Purified H101A following amylose exchange chromatography.

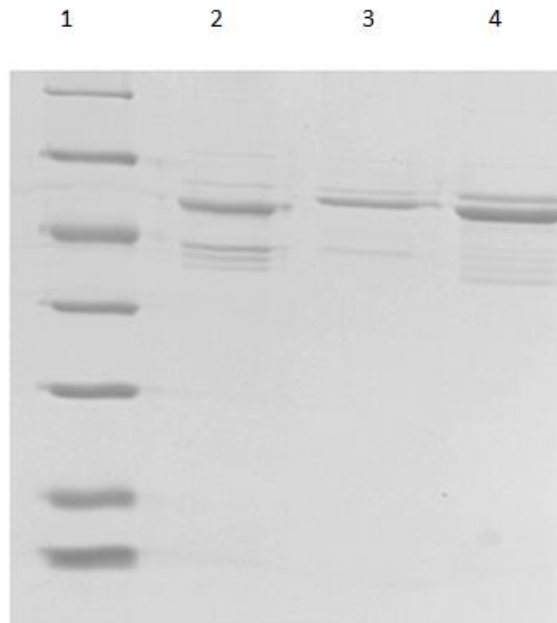


Figure 3.10: Purified MBP fused ghA, ghB and ghC following Amylose Resin chromatography Lane 1: Molecular marker, Lane 2: MBP-ghA, Lane 3: MBP-ghB, Lane 4: MBP-ghC

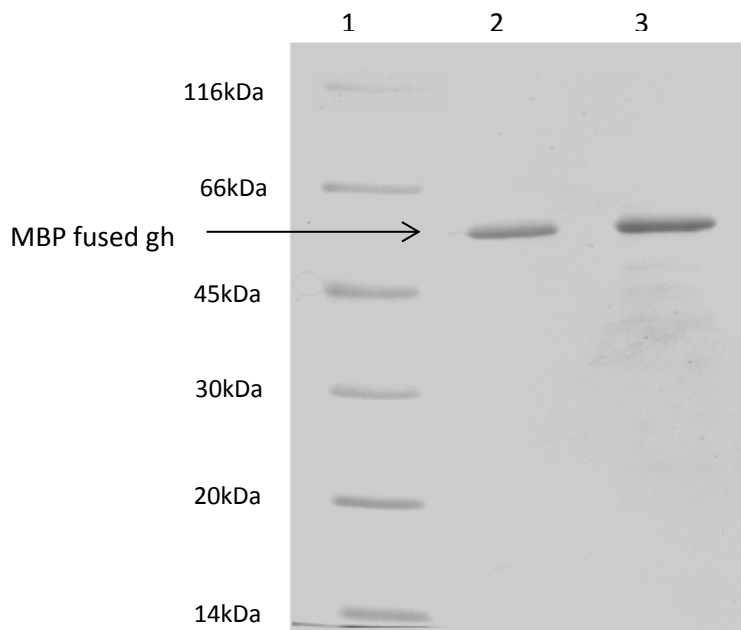


Figure 3.11: Purified MBP fused ghA-R162A and R162E following Amylose Resin chromatography Lane 1: Molecular marker, Lane 2: ghA-R162A, Lane 3: ghA-R162E,

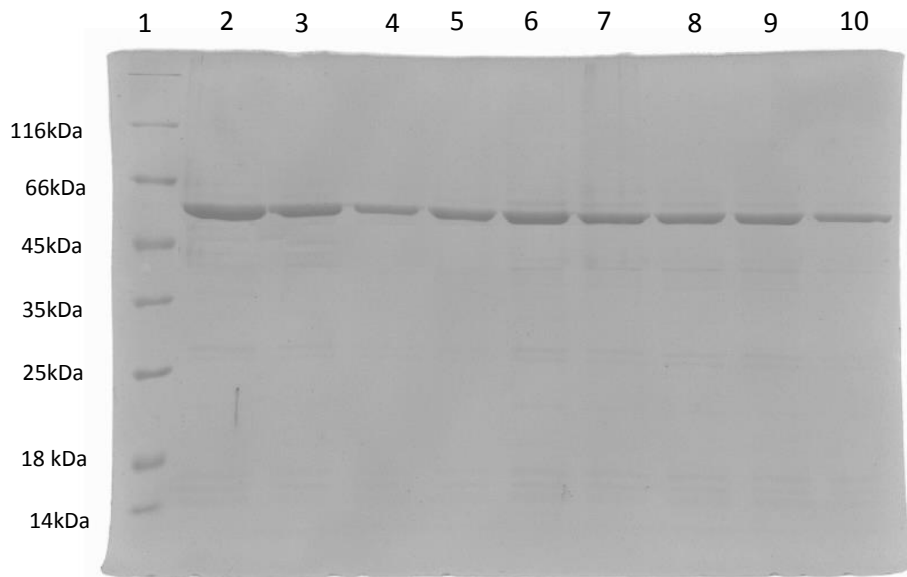


Figure 3.12: Purified MBP fused ghB mutants following Amylose resin and ion exchange chromatography: Lane 1: ghB-R114A, Lane 2: ghB-R114Q, Lane 3: ghB- R163A, Lane 4:ghB-R163E, Lane 5: ghB-R129A, Lane 6: ghB-R129E, Lane 6: ghB-T175L, Lane 7:ghB-H117D, Lane 8: Molecular marker

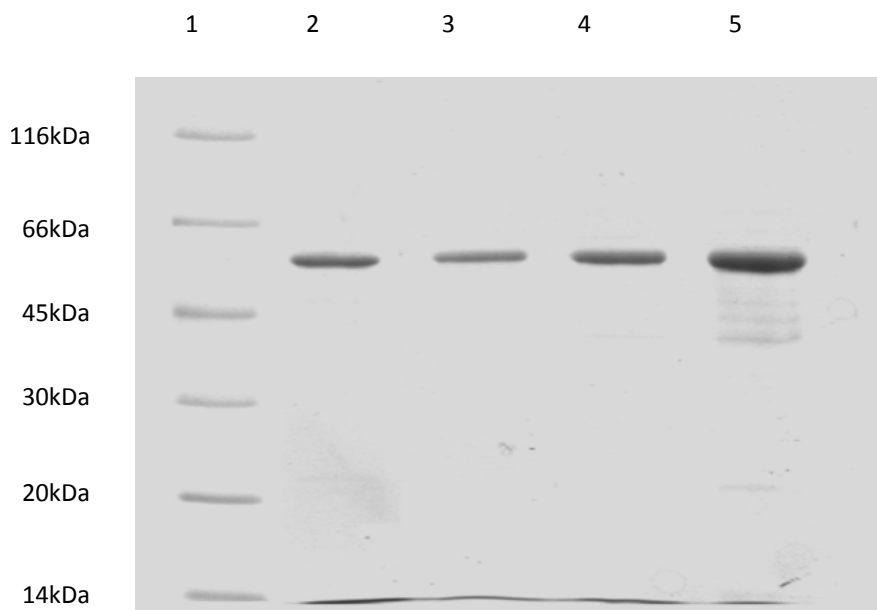


Figure 3.13: Purified MBP fused ghC mutants following Amylose resin chromatography: Lane 1: Molecular marker, Lane 2: ghC-R156E, Lane 3: ghC-L170E, Lane 4: ghC-H101D, Lane 5: ghC-R156E

Expression of human gC1qR in *E.coli* under T7 promotor system:

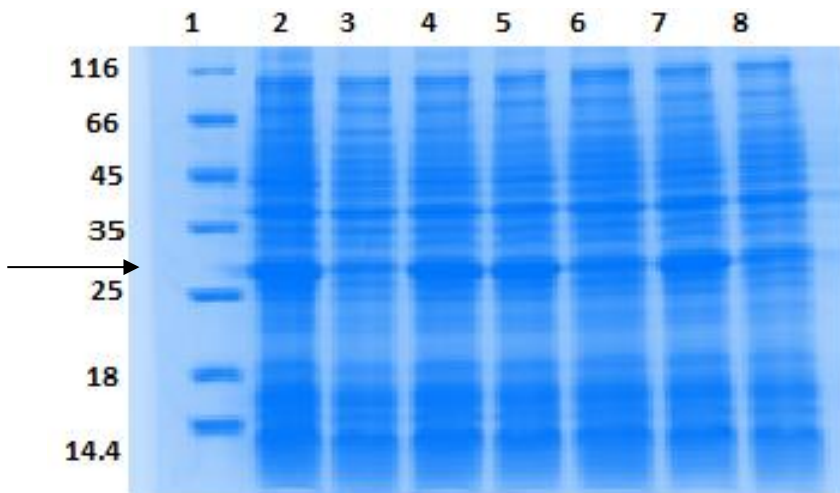


Figure 3.14: Expression of recombinant gC1qR following induction: gC1qR was transformed in *E. coli* BL21 and was induced with IPTG for 3 hours. A cell pellet of 100 μ l of bacterial culture was boiled under reduced conditions and loaded in each lane. Bacterial cells, grown in parallel but not induced with IPTG were used as a uninduced control. *Lane 1:* Molecular marker, *Lane 2, 4, 5 and 7:* Induced sample of gC1qR. *Lane 3, 7 and 8:* Uninduced sample of gC1qR. (15 % under reducing conditions).

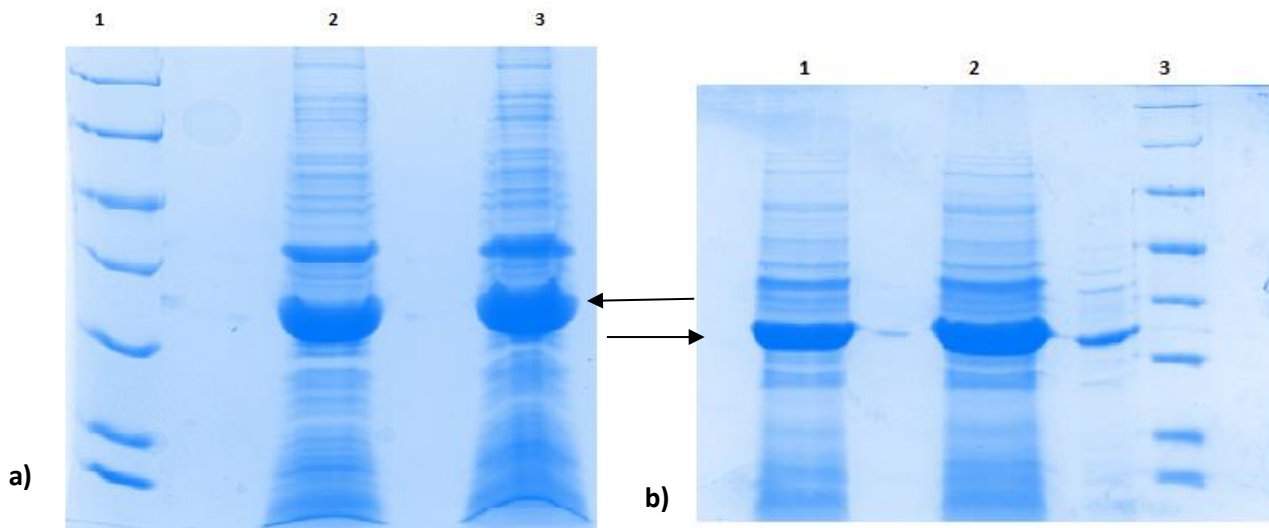


Figure 3.15: Supernatant of gC1qR after sonication. After observed expression following IPTG induction, lysis buffer was added to the protein pellet and samples were sonicated for 8 cycles for 30 seconds. Samples were spun down and supernatant was collected and run on a gel. **a)** *Lane 1:* Molecular marker. *Lane 2 and 3:* Supernatant of gC1qR. **b)** *Lane 3:* Molecular Marker *Lane 2 and 3:* Supernatant of gC1qR. Expression is shown under the 35kDa marker.

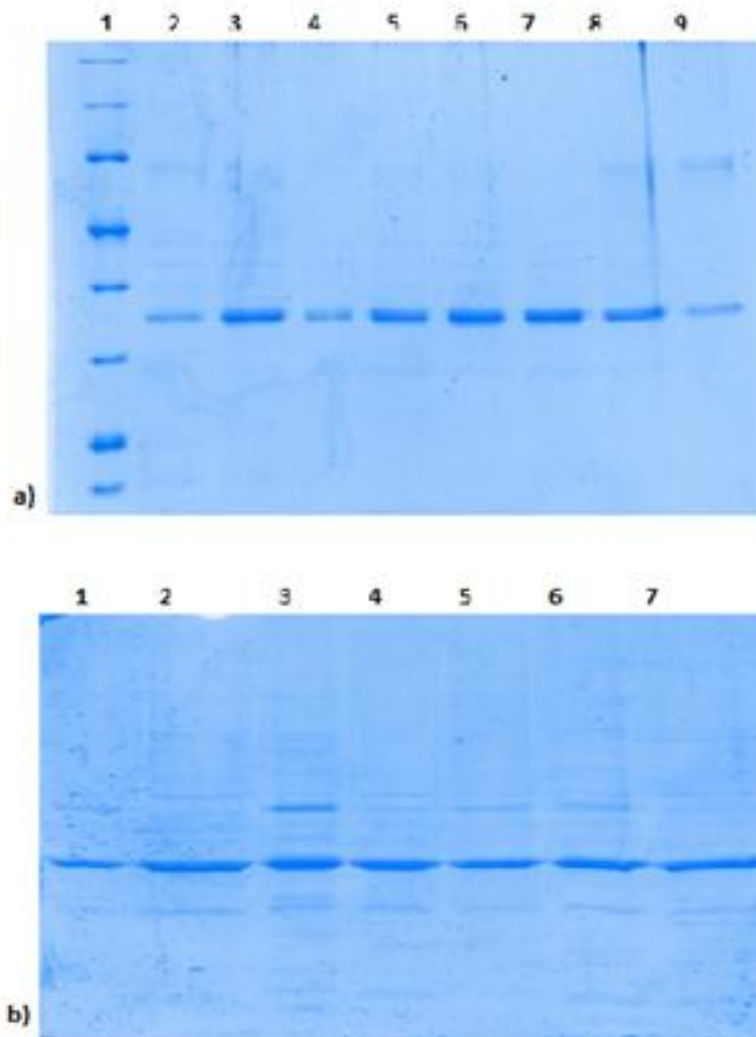


Figure 3.16: Purification of gC1qR: The gC1qR lysate was dialysed against 20mM Tris and 100mM NaCl at 4°C overnight. The supernatant was then spun at 4500 for 15 minutes and passed through a 5ml DEAE Sepharose column which was pre-equilibrated with 5 bed volumes of dialysis buffer. Purified gC1qR was eluted at a concentration of 450mM NaCl. Elutions were collected in 1ml fractions and each sample was run on a 12% SDS PAGE gel. **a)** Lane 1: Molecular Marker, Lane 2-9: Purified gC1qR fractions. **b)** Lane 1-7 Purified gC1qR fractions

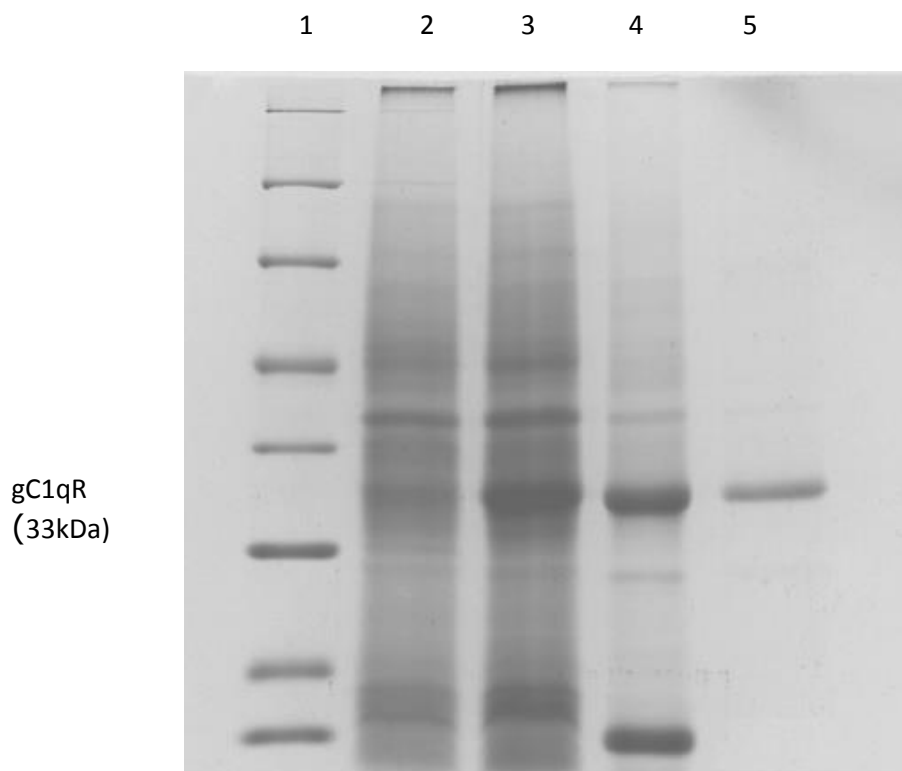


Figure 3.17: Expression and purification of 33kDa gC1qR. *E. coli* BL21 strain individually transformed with plasmid containing gC1qR was induced with IPTG for 3 hours and a cell pellet of 100 μ l of bacterial culture was boiled under reduced conditions (lane 2). Bacterial cells, grown in parallel but not induced with IPTG were used as an uninduced control (lane 1). After observed expression following IPTG induction, lysis buffer was added to the protein pellet and samples were sonicated for 8 cycles for 30 seconds. Samples were spun down and supernatant was collected and run on a gel (Lane 3.) The supernatant was then dialysed against 20mM Tris overnight and purified on a DEAE sepharose column, protein samples were eluted using 0.45M NaCl (lane 4). Expression was shown under the 35kDa marker Lane 1: Standard protein molecular marker. Lane 2: Uninduced expression of gC1qR, Lane 3: Induced expression of gC1qR, Lane 4: Soluble fraction after sonication, Lane 5: Eluted fraction of gC1q. 12% under reducing conditions



Figure 3.18: Biotinylated gC1qR: 2.2mg of biotin was dissolved in 400 μ l of H₂O. 3 μ l of this mixture was added per 100 μ g of protein and incubated at room temperature for 1 hour and dialysed against PBS to get rid of any excess biotin. The sample was then run on a gel to confirm. *Lane 1:* molecular marker, *Lane 2:* Biotinylated gC1qR. Expression is observed in lane 1 at 33kDa. (12% SDS-PAGE gel)

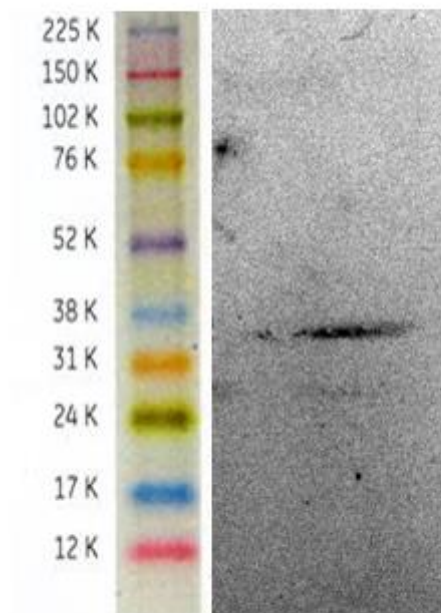


Figure 3.19 : Western Blotting to show purification of recombinant gC1qR: Expression and purification of recombinant gC1qR was verified by Western Blotting. 10 μ g of recombinant gC1qR protein was run on a 12% v/w gel and transferred for 1 hour at 320mA onto nitrocellulose membrane. Following blocking for 1 hour at room temperature with 5% non-fat milk in PBS, the membrane was probed with polyclonal antibody anti-gC1qR (1:5000). After this the membrane was washed 3 times in PBS-Tween 0.02%. PA-HRP was added (1:500) and colour was developed using DAB.

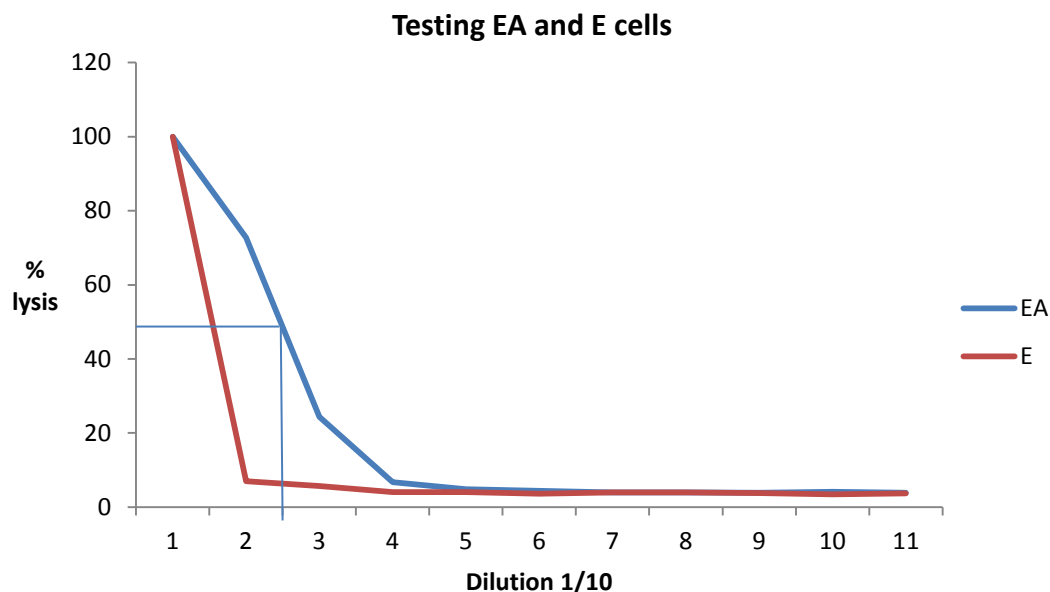


Figure 3.20: Hemolytic assay: 10^9 /ml sheep erythrocytes in DGVB⁺⁺ were washed 3 x in DPBS and sensitised (37° for 30 minutes) with a dilution of IgG anti sheep red blood cell stroma. After incubation the sensitised EA's were pelleted, washed 1x in DPBS and 3x in DGVB and re-suspended in the same buffer. E cells were prepared in parallel without the addition of haemolysin to use as a control. The EA's were then tested by incubating, (37° for 1 hour) 100µl of EA's with 100µl of a 1:10 dilution (double diluted down) of NHS in DGVB. The percentage lysis of the EA and E cells was calculated and plotted, and the ch50 value was obtained at observing the dilution of serum that gave 50% lysis.

3.4.3 Determination of CH₅₀ value of the NHS

The dilution of serum to use for complement assays was determined by a CH₅₀ value test. This test would determine the dilution of serum needed by testing the functional capabilities of the serum complement components to cause 50% lysis of the SRBCs, pre sensitised with haemolysin. Upon lysis, the haemoglobin released is measured in this assay. When sensitised SRBCs were incubated with serum, the classical pathway was activated leading to the lysis of the SRBCs. By looking at the graph we were able to see that a serum dilution of 1/30 was sufficient to create 50%. E cells were used as a negative control to confirm sensitisation of the EA cells.

Recombinant gC1qR activates complement in solution and solid phase

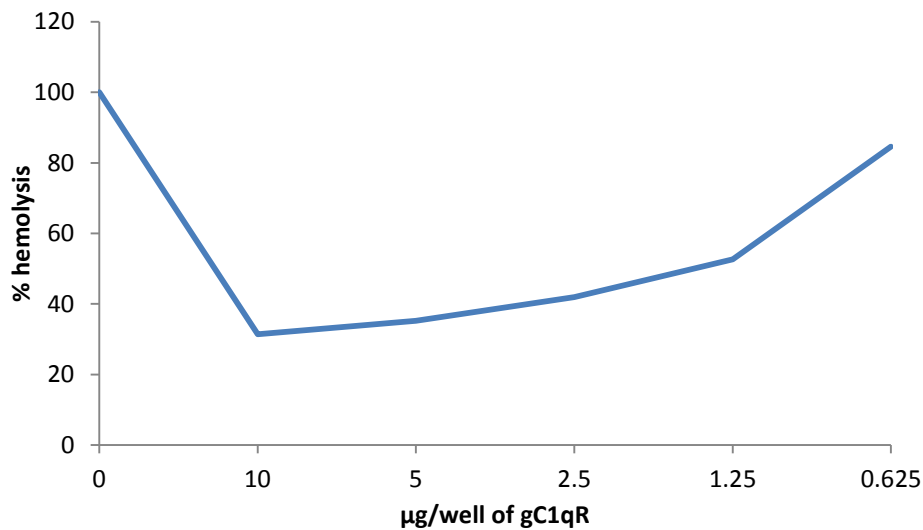


Figure 3.21: Recombinant gC1qR activates complement in solid phase: gC1qR was coated on microtitre wells in carbonate buffer (100µl) at different concentrations, (10, 5, 2.5, 1.25 and 0.625µg/well) and the plate was incubated overnight at +4 C. Contents were discarded and 1:40 dilution of NHS in DGVB++ (total volume of 100µl) was added to each well and the plate was incubated at 37°C. Following this, the wells were washed three times with DGVB++ and 100µl of EA cells (1×10^8) were added to each well. This was then incubated for a further hour. After this, 180µl of each sample was collected in an eppendorf tube and spun down for 5 minutes at 5000rpm. 100µl of the supernatant was read under 520nm wavelength. H₂O and EA cells were used for 100% lysis.

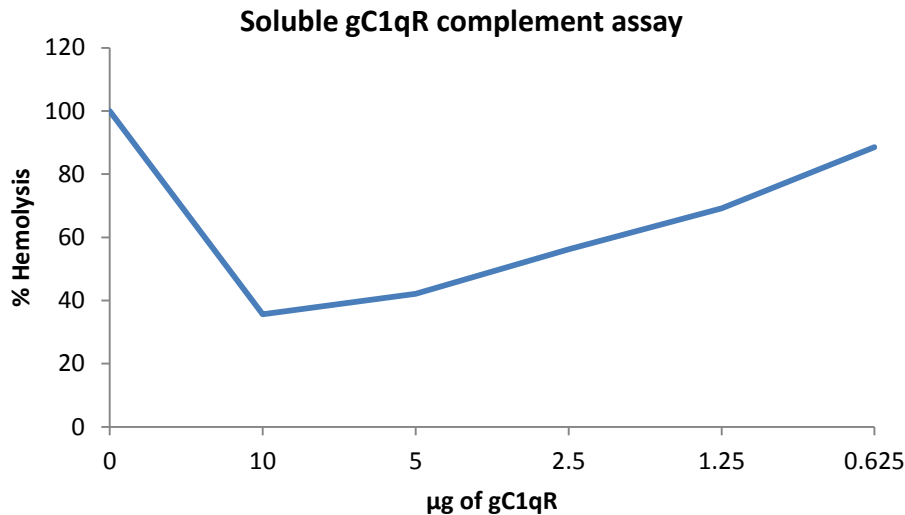
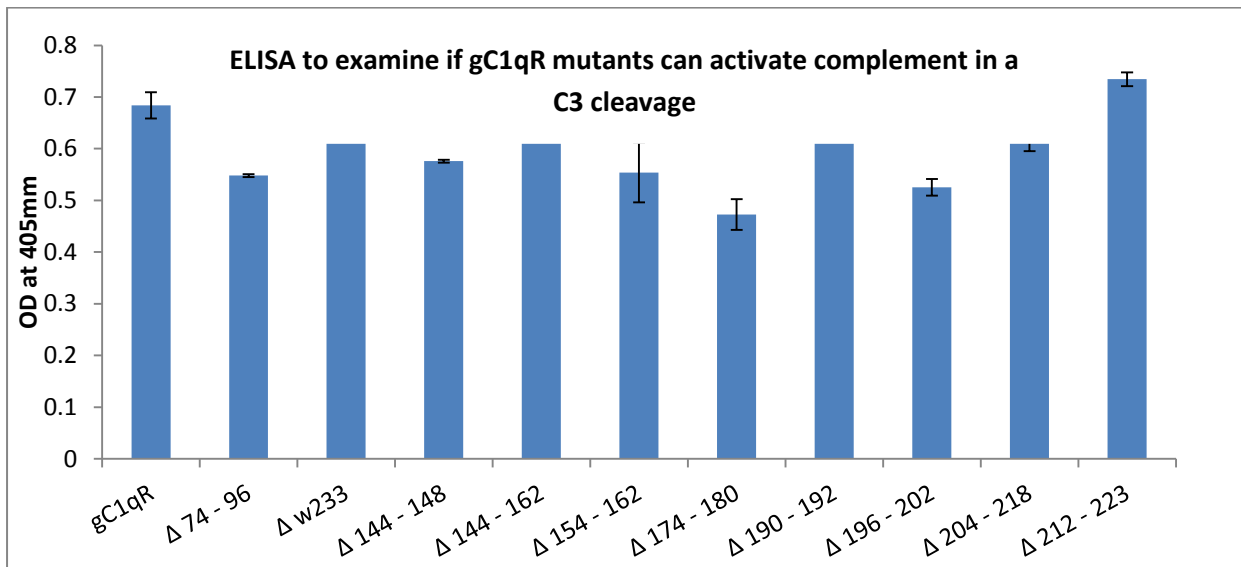
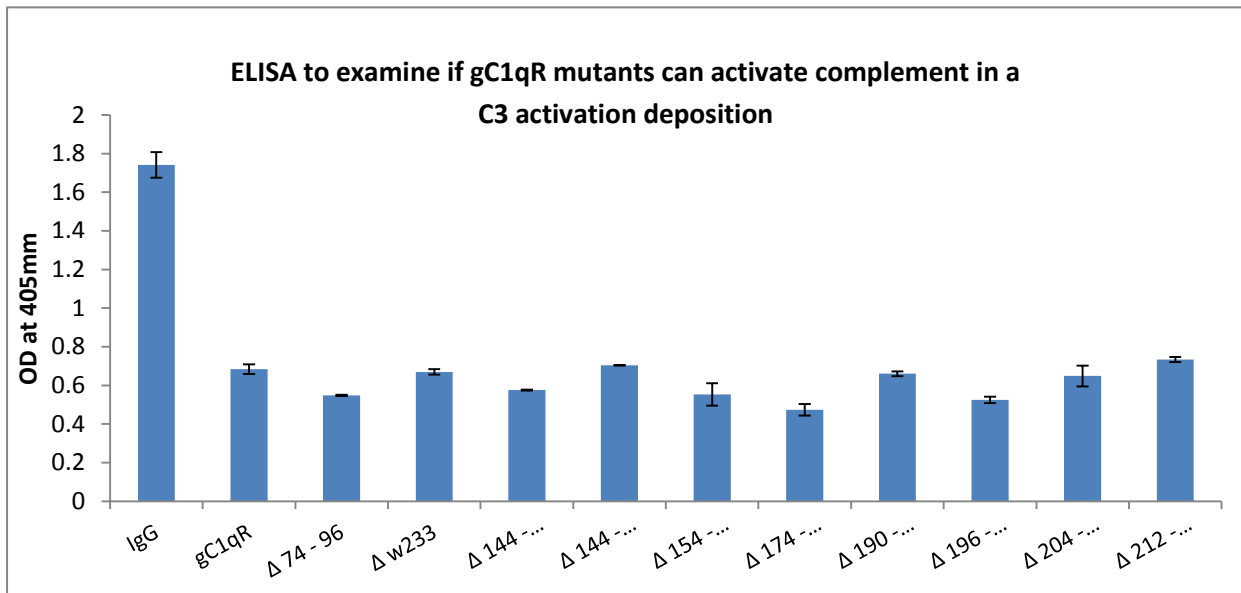


Figure 3.22: Recombinant gC1qR activates complement in solution phase: gC1qR was incubated for 1 hour at 37°C at different concentrations, (10, 5, 2.5, 1.25., 0.625µg) with NHS in DGVB⁺⁺ (at a dilution of 1:40) to give a total volume of 100µl. Following this, 100µl of EA cells (1×10^8) were added to each sample. This was then incubated for a further hour. After this, samples were spun down for 5 minutes at 5000rpm. 100µl of the supernatant was read under 520nm wavelength. H₂O and EA cells were used for 100% lysis.

3.4.4 Recombinant gC1qR and deletion mutants activate complement

Previously, gC1qR has been reported to activate complement. Thus, studies were performed to evaluate whether gC1qR generated using T7 construct was able to activate the classical pathway. Figure 3.21 demonstrates gC1qR incubated in solid phase on microtitre wells at different concentrations (10-0.625µg/well) of NHS activated complement in a dose dependent manner as the concentration of gC1qR decreased. Similar to this, Figure 3.22 shows an identical assay carried out with the exception of gC1qR (at the same concentrations) incubated in solution with NHS. Again, consistent with the previous complement assay, gC1qR in solution was also able to activate complement in a similar dose dependant way. Both figures show that 10µg of gC1qR bought C1q mediated haemolysis down by 75%. The availability of gC1qR deletion mutants allowed us to determine whether gC1qR with various deleted residues are able to activate complement in solid phase. Complement activity was detected using anti C4d and IgG was used as a positive control. Our assay showed that all the gC1qR deletion mutants retained their ability to activate the classical pathway.

a)



b)

Figure 3.23: ELISA to examine if gC1qR deletion mutants activate complement: a) Compared with IgG as a positive control, b) Data shown without IgG. Wells were coated with 4μg/ml of each gC1qR deletion mutant in coating buffer and incubated at 37°C for two hours. Contents were discarded and wells were blocked for 1 hour for 2% BSA. Wells were washed 3 times with DGVB⁺⁺ and 100μl of 1/10 dilution of NHS in DGVB⁺⁺ was added to each well and incubated for 1 hour at 37°C. The wells were repeatedly washed in DGVB⁺⁺ and anti-C4d (1:2000) was added in DGVB⁺⁺ and incubated for a hour at 37°C. Bound C4d was detected with AP Neutravidin and developed using pNPP.

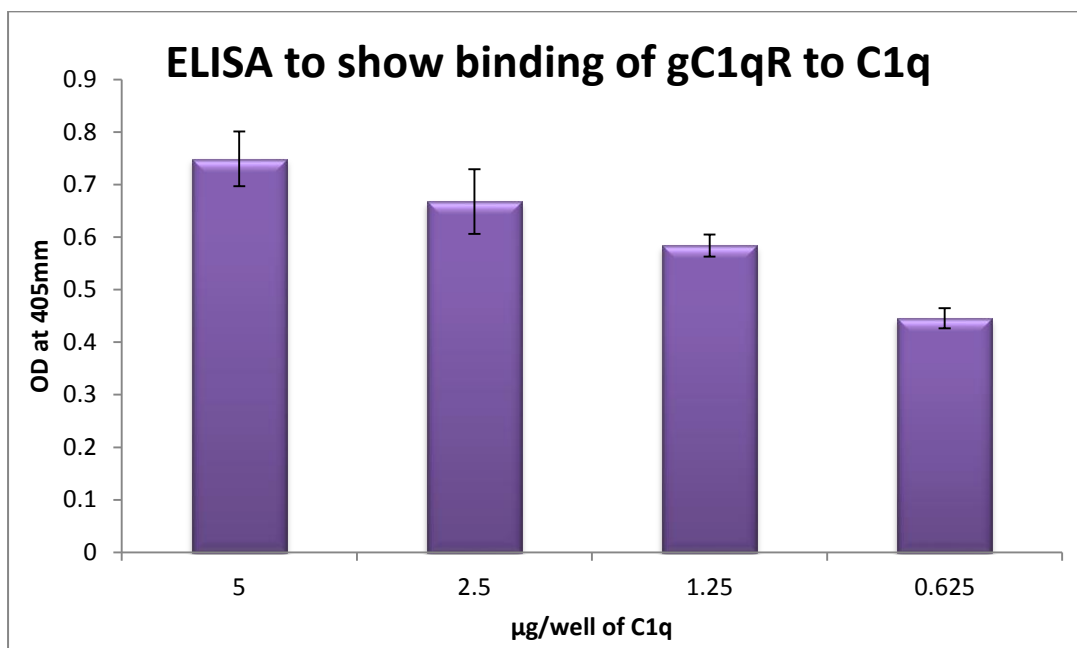


Figure 3.24: ELISA to show binding of gC1qR to C1q: Microtitre wells were coated in duplicates with different concentrations of C1q, (5, 2.5, 1.25 and 0.625µg/well) and incubated overnight at 4°C. Following morning contents were discarded and wells were blocked with 2%BSA in PBS for 1 hour at 37°C. Wells were then washed with PBS + 0.05% Tween three times and 2.5µg of biotinylated gC1qR was added per well in Calcium buffer and incubated for a further 1 hour at 37°C. Following repeated washes, bound gC1qR was detected by streptavidin HRP (1:5000) in PBS. Wells were washed again and the colour was developed using OPD tablets.

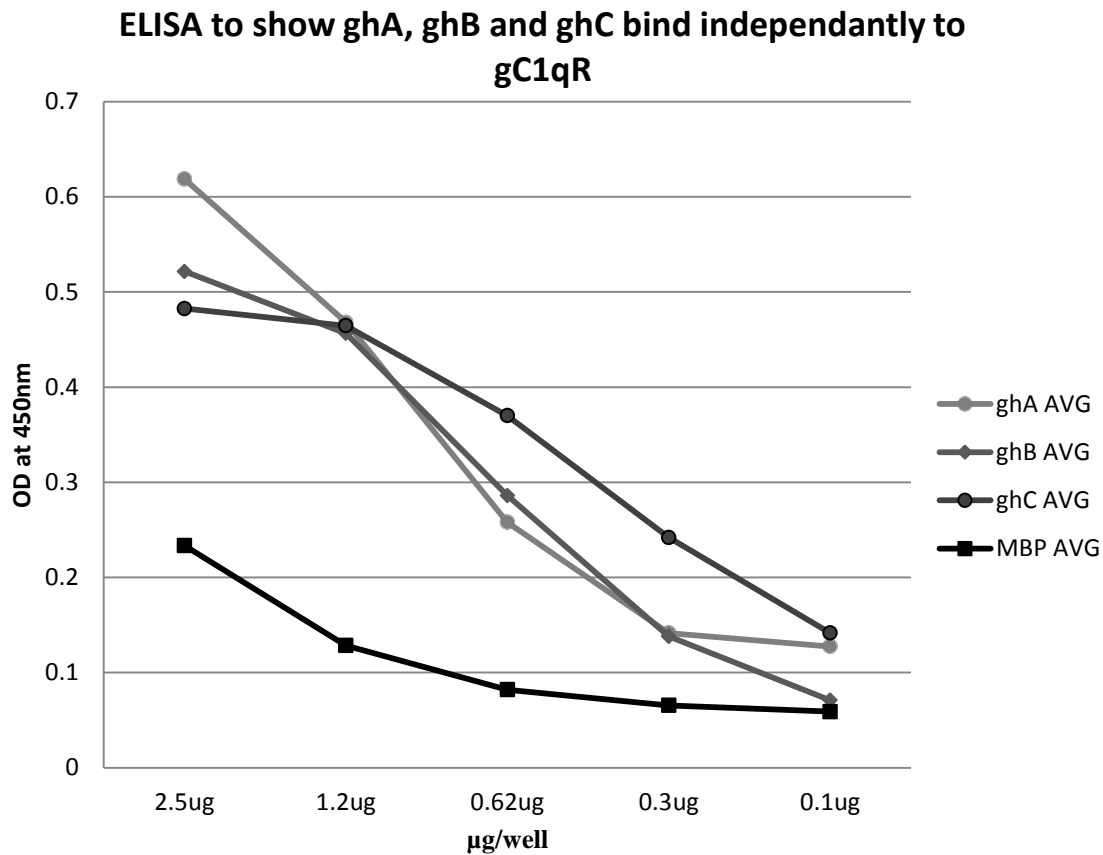


Figure 3.25: ELISA to assess binding between the globular head receptor gC1qR and ghA, ghB, ghC and MBP. gC1qR was coated in coating buffer, Ph 9.6 at a concentration of 1ug/well. 100µl of this was coated onto microtitre wells and left overnight at 4 degrees. The next morning wells were discarded and blocked with PBS containing 2% BSA for 3 hours. After 2 washes in PBS-T, the wells were incubated with wild-type forms of ghA, ghB, ghC and MBP as a negative control in a calcium buffer (1M Tris pH 7.5, 0.1M CaCl₂ and 5M NaCl) at different concentrations (2.5, 1.2, 0.62, 0.3, 0.1µg/well) for 1.5 hours at 37 degrees and 1.5 hours at 4 degrees. Following washing, bound gh protein was detected using mAb anti-MBP as a primary antibody and goat-anti mouse HRP conjugate as the secondary antibody

3.4.5 gC1qR binds differentially to three globular head modules of human C1q

To confirm that gC1qR is the globular head receptor for C1q, binding was measured by performing a direct binding ELISA. When biotinylated gC1qR was added to C1q coated on microtitre wells, there was a concentration dependent increase clearly indicating that gC1qR bound to C1q. (Figure 3.24)

Different concentrations of the recombinant ghA, ghB and ghC were allowed to bind a constant concentration of gC1qR, following probing with the goat anti mouse conjugated to HRP, MBP was used as a negative control. As shown in figure 3.25, ghA,

ghB and ghC modules all bound to gC1qR independently, in a dose dependent manner. Figure 3.25 shows that ghA showed stronger binding at a concentration of 2.5 μ g when compared with the other two modules. However the change in concentration from 1.2 μ g showed ghC to increase its binding capacity to gC1qR when compared with ghA and ghB. The fact that ghA has shown to bind to gC1qR at a higher affinity at a higher concentration is consistent with previous reports by Ghebrehiwet et al, 1996 that has implicated ghA to be the most important globular head in the C1q-gC1qR interaction. To confirm that each globular head bound independently to gC1qR, far western blots were performed using the expressed and purified gC1qR, ghA, ghB and ghC. By transferring ghA, ghB and ghC onto PDVF membrane and probing with gC1qR, we were able to see three bands as shown in figure 3.27. Figure 3.26 shows gC1qR transferred onto PDVF membrane and individually adding ghA, ghB and ghC to each blot. The appearance of three strong bands validated our previous findings that each globular head binds specifically to gC1qR.

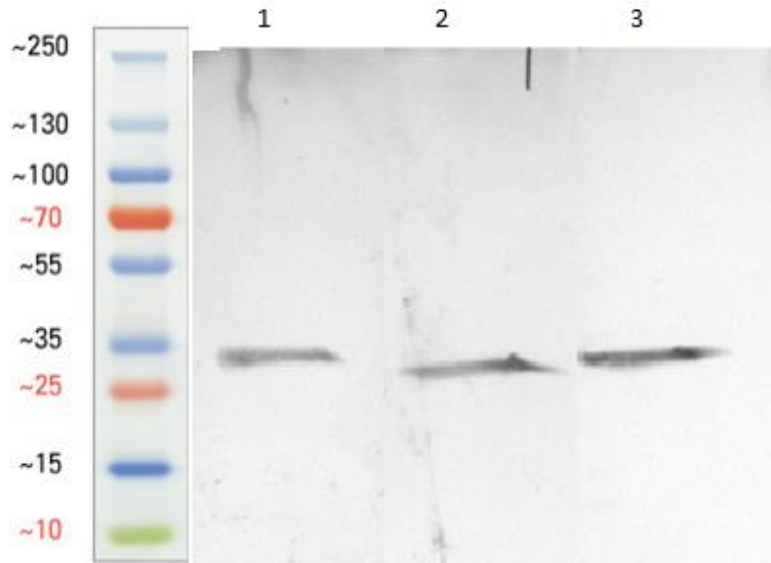


Figure 3.26: Far Western Blot to show ghA, ghB and ghC bind independantly to gC1qR: 15 μ g of gC1qR was loaded on a gel in three wells and run. The gel was cut into three sections and transferred onto PDV membrane for 1 hour. The membrane was also cut into three sections and blocked with 5% non-fat milk in PBS for 2 hours. 10 μ g of ghA, ghB and ghC in PBS were added to each respective memnbrane strip and incubated overnight at room temperature. The membranes were then washed 3 times in PBS-Tween for 20 minutes each and 10 μ l of mAb anti-MBP in 2% non fat milk was added to each membrane and incubated for 2 hours at 37 degrees. Following washing 3 times in PBS-Tween for 20 minutes each, 10 μ l of IgG-HRP in 2% non fat milk was added and incubated for 1 hour at room temperature. The bands were developed using DAB tablets dissolved in water. *Lane 1:* gC1qR-ghA interaction, *Lane 2:* gC1qR-ghB interaction. *Lane 3:* gC1qR-ghC

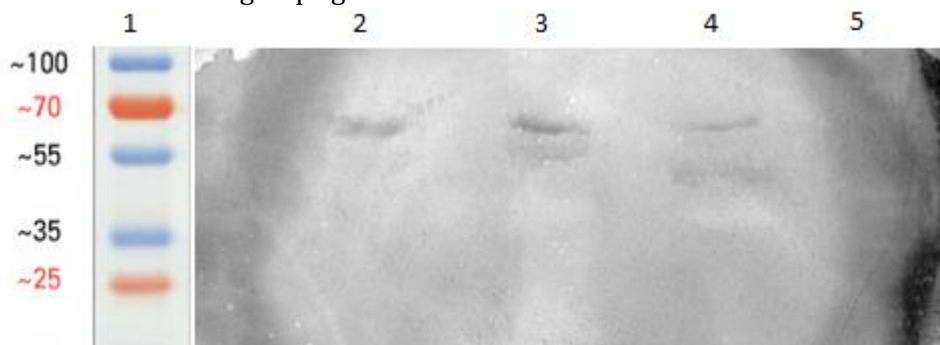


Figure 3.27: Far Western Blot to show gC1qR binding to ghA, ghB and ghC modules: 10 μ g of ghA, ghB, ghC and MBP was run on a gel and transferred onto a PDVF membrane for 1 hour at 320mA. Following this, the membrane was blocked for 1 hours with 5% non-milk fat powder in PBS. 15 μ g of gC1qR in PBS was added to the membrane and incubated overnight. The next morning the membrane was washed 3 times for 20 minutes each with 0.02% PBS-T. Anti-gC1qR (1:1000) was added in 2% non-fat milk in PBS and the membrane was incubated for 2 hours at 37 $^{\circ}$ C. The bands were detected using Protein A-HRP conjugate (1:1000) in PBS and bands were developed using DAB tablets. *Lane 1:* Molecular marker *Lane 2:* ghA bound to gC1qR, *Lane 3:* ghB bound to gC1qR, *Lane 4:* ghC bound to gC1qR, *Lane 5:* MBP (negative control)

3.4.6 gC1qR binds to ghA, ghB and ghC independently on far western blot

A far western blot was performed to examine the interaction between ghA, ghB ghC and gC1qR. We examined the binding of the individual globular heads to gC1qR (figure 3.26) and the binding of gC1qR to the globular heads (figure 3.27). Both blots revealed that ghA, ghB and ghC were able to independently bind to gC1qR on PVDF membrane and gC1qR was able to interact with all three globular head modules transferred onto the membrane which further validated the gC1qR-C1q interaction.

3.4.7 ghA R162 is crucial for C1q-gC1qR interaction

Using ELISA, we examined the gC1qR binding ability of recombinant ghA and its single residue mutants ghA-R162A and R162E. Different amounts of gC1qR were coated on microtitre wells, incubated with wild-type ghA, R162A and R162E, washed and probed with goat anti mouse HRP conjugate. As shown in figure 3.28, substitution of Arg 162 to Alanine (R162A) resulted in ~ 80% reduction in gC1qR binding. Similarly substitution of Arginine 162 with glutamate (R162E) resulted in similar abrogation of binding when compared to wild type ghA. To further confirm these observations, we carried out a far western blot, (Figure 3.29) to verify whether the Arg¹⁶² residue is specific in the C1q-gC1qR interaction. Figure 3.29 shows a clear band for wild type ghA. However, probing with the mutants yields results consistent with ELISA data. A very faint band was observed for R162A and no band detected on the blot for R162E.

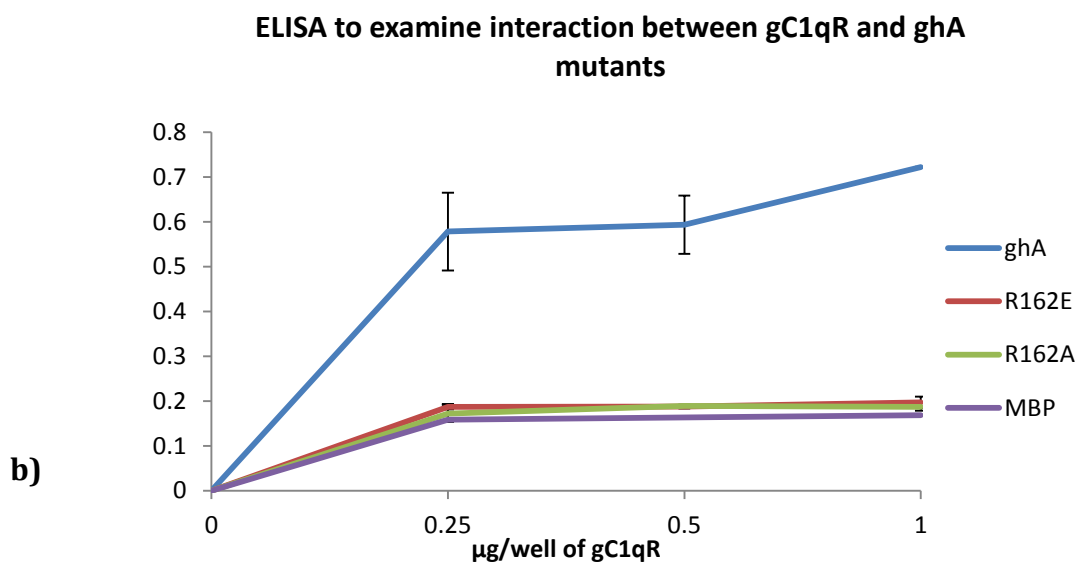
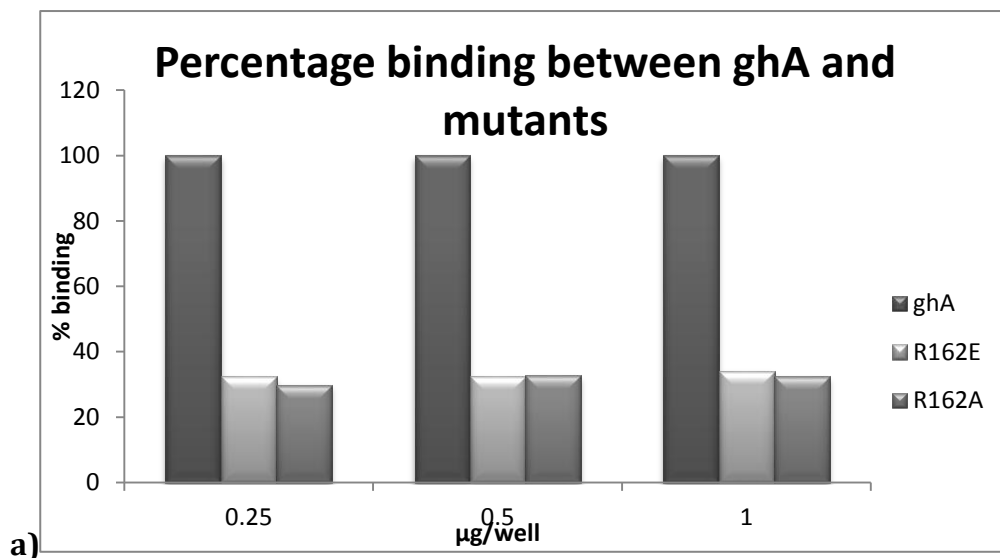


Figure 3.28: Percentage binding to show inhibition of ghA mutants to gC1qR. B) ELISA to show interaction of ghA mutants with gC1qR: Microtitre wells were coated in duplicates with different concentrations (1, 0.5, 0.25µg/well) of gC1qR in carbonate buffer and incubated at +4 overnight. Contents were discarded and wells were blocked for 2 hours with 2% BSA at 37°. Following washing with PBS + 0.05% Tween, 2.5ug/well of ghA wild type, R162A, R162E and MBP was added and the plate was incubated for 1.5 hours at 37° and 1.5 hours at +4. Wells were washed bound protein was detected using anti-MBP (1/5000) and IgG-HRP (1:5000) in PBS. Colour was developed using OPD buffer. The plate was read at a wavelength of 450nm. **A)** The percentage inhibition binding of the ghA mutants to gC1qR was worked out by using wild type ghA binding as 100% positive control

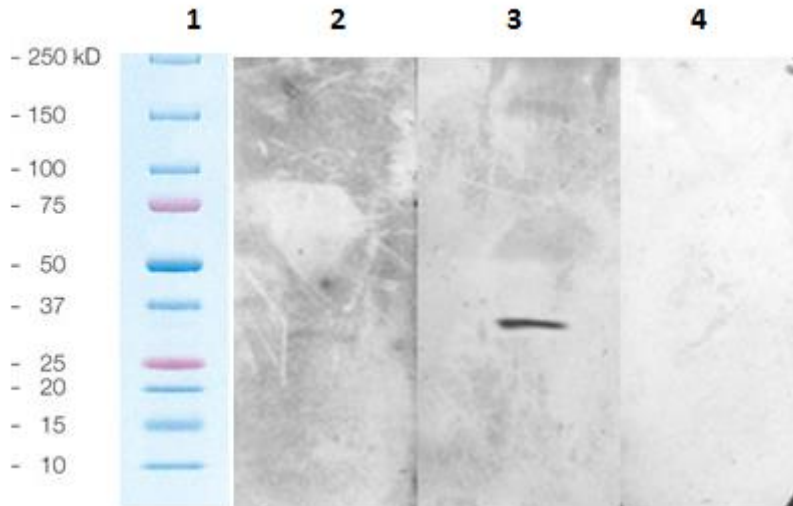


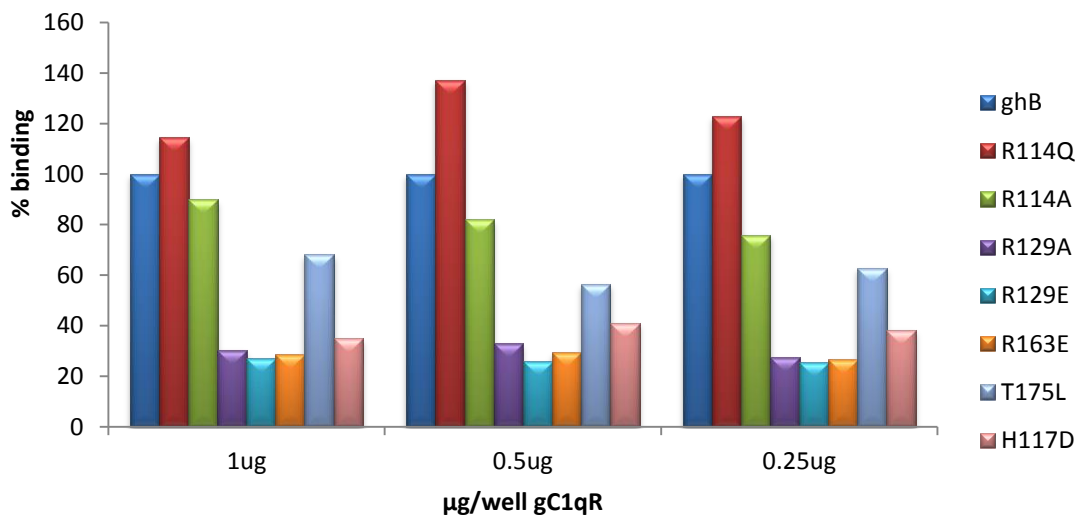
Figure 3.29: Far Western Blot to show lack of binding of ghA mutants R162A and R162E to gC1qR: 10µg of gC1qR was run in three lanes on a 15% SDS gel and transferred for 1 hour at 320mA onto PDVF membrane. The membrane was blocked for 1 hour in 5% non-fat milk powder in PBS at room temperature. Next, the blot was cut into three pieces to separate the transferred gC1qR protein. Each strip was then probed with 50µg in 10ml of either WT ghA, R162E or R162A in Calcium buffer. This was incubated overnight at room temperature. The blots were washed three times in PBS + 0.02% Tween for 10 minutes each on a shaker. Next, the primary antibody mAb anti-MBP was added in PBS (1:1000) and left to incubate for 2 hours at 37°C. The membranes were repeatedly washed and the secondary antibody IgG-HRP was diluted in PBS (1:1000) and incubated for 1 hour at room temperature. Following repeated bands were developed using DAB tablets *Lane 1:* Molecular marker, *Lane 1:* gC1qR probed with R162A. *Lane 2:* gC1qR probed with WT ghA. *Lane 3:* gC1qR probed with R162E.

3.4.8 R114Q appears important for stabilising C1q-gC1qR interaction

Using ELISA the ability of ghB and its single residue mutants R114Q, R114A, R163A, R163E, T175L, R129A, R129E and H117D to bind gC1qR was examined. gC1qR was coated on microtitre wells and probed with recombinant mutational forms of MBP-ghB. Results showed that substituting Arg¹¹⁴ to Glutamine (R114Q) bound with a greater affinity to gC1qR than the wild type ghB (Figure 3.30). Figure 3.30a shows that this substitution increased ghB binding to nearly 30% greater than the wild type ghB when the concentration of gC1qR increased from 0.5µg to 1µg, suggesting that the insertion of a polar amino acid such as glutamate is able to enhance binding between the two proteins. Substituting Arg¹¹⁴ with Alanine, however, was not comparable with R114Q as figure 3.30a shows a minimum of 40% difference in binding between the two

mutants to gC1qR. Out of all the ghB mutants, R163E showed the most dramatic drop in gC1qR binding than the wild type ghB, with R163E inhibiting binding up to 65% at the highest concentration of gC1qR (1 μ g). R129A and R1219E showed similar binding abilities as figure 3.30a again shows a drop of over 70% compared to wild type ghB pointing out that Arginine residues play a role in this interaction. Binding of H117D was comparable to this. Calculating from the graph, T175L was shown to inhibit binding up to 35% and H117D dropped to 65%. This shows that substituting Arginine with glutamate has an adverse effect on the binding of gC1qR as binding was shown to be nearly abolished. Substituting His to Asp reduced ghB affinity for gC1qR by nearly 70%, when compared to the wild type ghB, which suggests that His¹¹⁷ is important in gC1qR binding. Comparing the ghB deletion mutants, the most significant effect observed on gC1qR binding was the role of mutants R136E, T175L and H117D suggesting that Arg, Tyr and His at these points are also crucial for gC1qR binding.

Percentage Binding between ghB and its mutants with gC1qR



a)

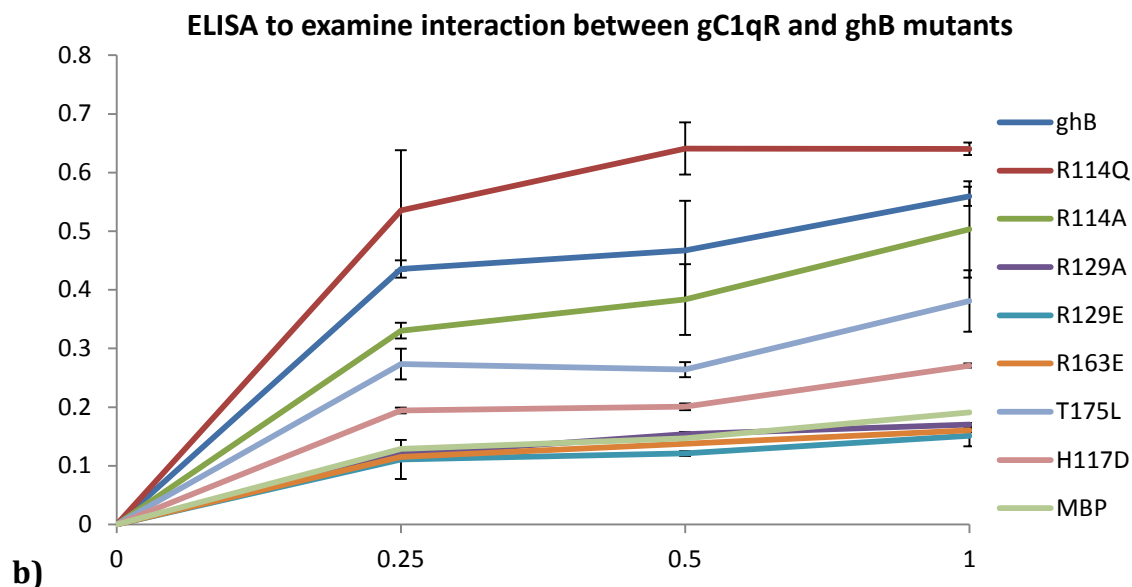
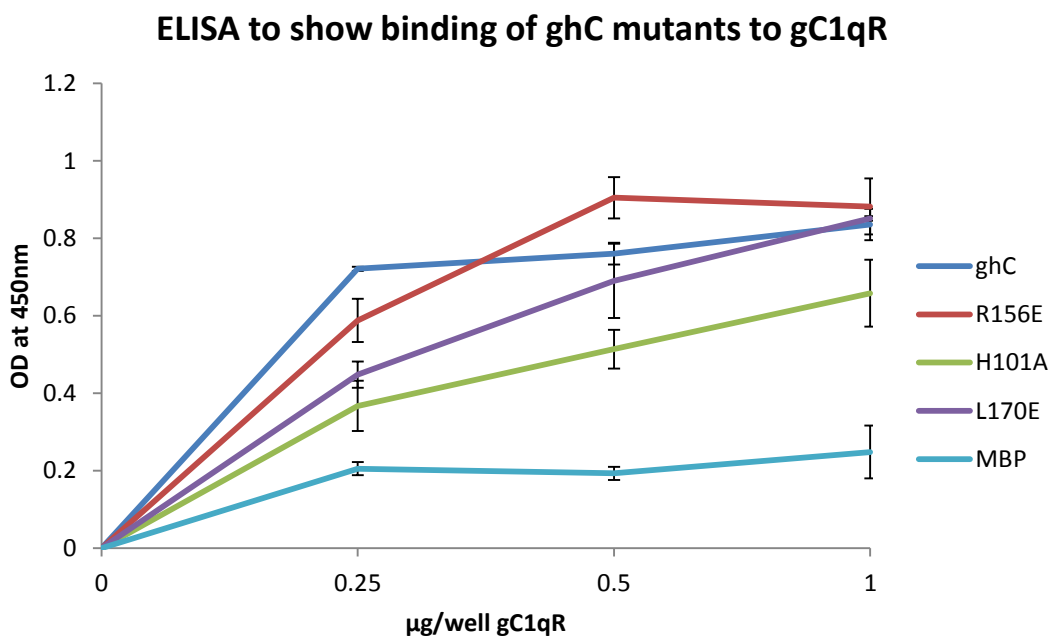
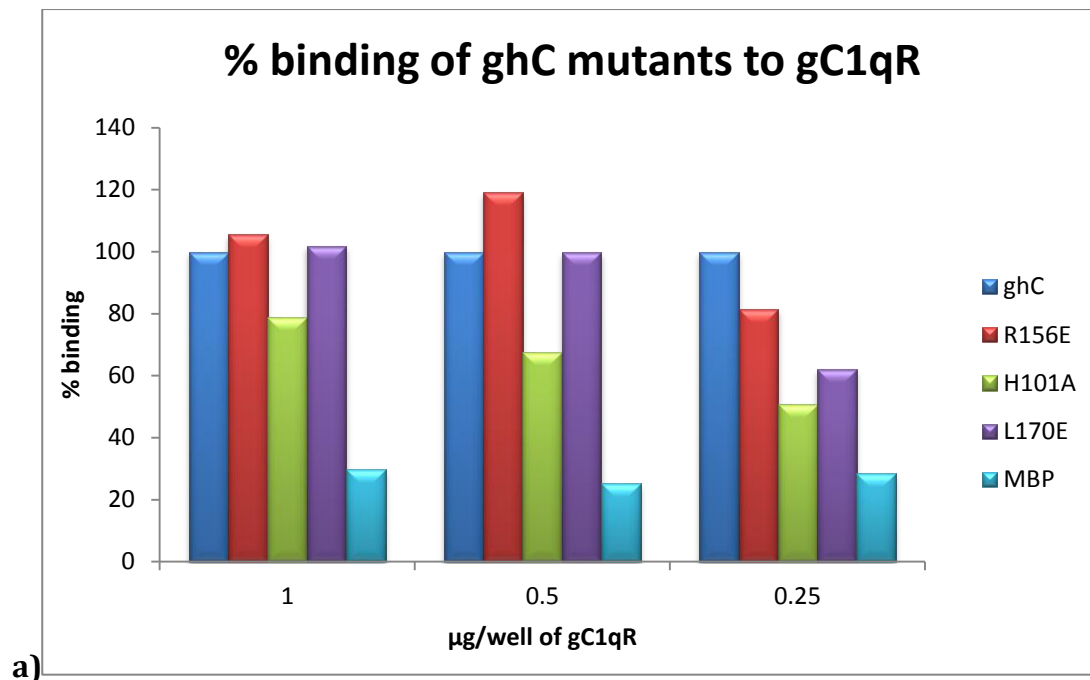


Figure 3.30: a) Percentage binding to show inhibition of ghB mutants to gC1qR. b) ELISA to show interaction of ghB mutants with gC1qR. **a)** The percentage inhibition binding of the ghB mutants to gC1qR was worked out by using wild type ghB binding as 100% positive control. **b)** Microtitre wells were coated in duplicates with different concentrations (1, 0.5, 0.25µg/well) of gC1qR in carbonate buffer and incubated at +4 overnight. Contents were discarded and wells were blocked for 2 hours with 2% BSA at 37°. Following washing with PBS + 0.05% Tween, 2.5ug/well of ghB wild type, R114Q, R114A, 129A, R129E, R163E, T175L, H117D and MBP was added and the plate was incubated for 1.5 hours at 37° and 1.5 hours at +4. Wells were washed bound protein was detected using anti-MBP (1/5000) and IgG-HRP (1:5000) in PBS. Colour was developed using OPD buffer. The plate was read at a wavelength of 450nm

3.4.9 Contributions of ghC substitutions in gC1qR binding

Using ghC substitution mutants we were able to examine the ability of R156E, H101A and L170E to interact with gC1qR. Our ELISA result revealed that all three substitution mutants were able to bind to gC1qR in a dose-dependent manner (figure 3.31b). The substitution of Arg¹⁵⁶ to Gln revealed that this had no effect in gC1qR binding. R156E was able to interact with gC1qR in a similar manner as the WT ghC. The ghC mutant, L170E, revealed that by substituting Leu¹⁷⁰ with Gln slightly reduced its affinity to gC1qR by 13%. A greater reduction of binding (35%) (figure 3.31a) was observed when His¹⁰¹ was replaced with Ala, suggesting that this residue of the ghC chain is involved in the C1q-gC1qR interaction.



b)

Figure 3.31: a) Percentage binding to show inhibition of ghC mutants to gC1qR. b) ELISA to show interaction of ghC mutants with gC1qR: a) The percentage inhibition binding of the ghC mutants to gC1qR was worked out by using wild type ghC binding as 100% positive control b) Microtitre wells were coated in duplicates with different concentrations (1, 0.5, 0.25µg/well) of gC1qR in carbonate buffer and incubated at +4 overnight. Contents were discarded and wells were blocked for 2 hours with 2% BSA at 37°. Following washing with PBS + 0.05% Tween, 2.5ug/well of ghC wild type, R156E, H101A, L170E and MBP was added and the plate was incubated for 1.5 hours at 37° and 1.5 hours at +4. Wells were washed bound protein was detected using anti-MBP (1/5000) and IgG-HRP (1:5000) in PBS. Colour was developed using OPD buffer. The plate was read at a wavelength of 450nm

3.4.10 Identification of additional C1q binding sites on gC1qR

Interestingly, deletion of the previously identified C1q binding site 74-96 appeared to support C1q binding as well as WT gC1qR (Figure 3.32) suggesting that there are other residues on gC1qR that are key players in the interaction with C1q. However, additional domains were identified that drastically reduced C1q binding when deleted. These include residues 190-192, 196-202 and 202-218. The fact that residues 190-192 and 196-202 are essential for trimer formation of gC1qR, indicates that monomeric gC1qR does not bind efficiently to C1q. HK has been found to bind to gC1qR at residues 204-218 and activate the KKS system, and our data suggests that this stretch of 14 residues also provides an additional site for C1q, pointing out that these plasma proteins could potentially share overlapping sites on gC1qR. Deleting the single amino acid W233G seemed to have no effect in reducing C1q binding suggesting that this protruding residue has no involvement in the C1q-gC1qR interaction.

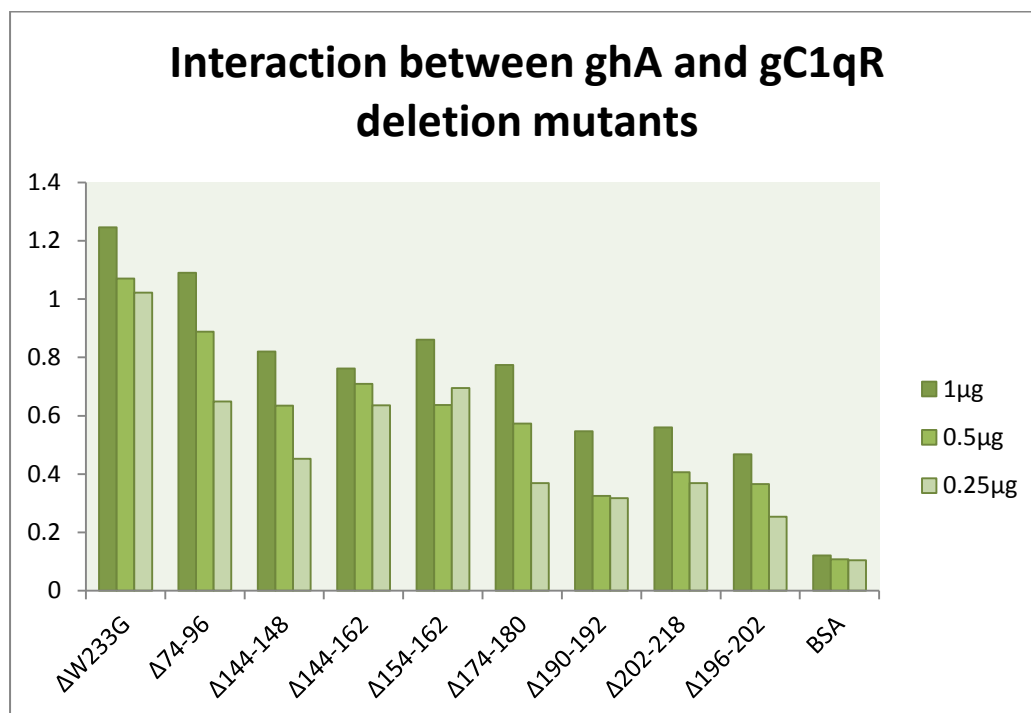


Figure 3.32: ELISA to identify additional binding sites for C1q on gC1qR. Duplicate wells of microtiter plates were first coated with 100 µl of wild type or gC1qR mutants (Δ gC1qR) in carbonate buffer at different concentrations, (1, 0.5, 0.25 µg/well) and incubated overnight at 4°C. Contents were discarded and the wells were blocked with 2% BSA in PBS for 2 hours at

37°C. The wells were then washed three times in PBS +0.05% Tween and wild type ghA (2.5µg/well) was added in Calcium buffer. The plate was incubated for 1.5 hours at 37°C and 1.5 hours at 4°C. Following repeated washes the bound wild type globular head was detected by 1:5000 dilution of mAb anti-MBP in PBS, followed by IgG-HRP conjugate (1:5000) in PBS. Wells were developed using OPD tablets

3.4.11 Concomitant increase of gC1qR and C1q expression in adhesive monocytes

In order to investigate the pattern of expression of C1q and gC1qR, an assay was devised using monocytes. The monocytes were derived from fresh blood and maintained in culture in order to become adherent. The adherence phenotype was observed using microscopy during the time points analyzed (results not shown) outlined in Figures 3.33 and 3.34. The adherence phenotype *in vitro* simulates the adherence of monocytes to endothelial surfaces *in vivo* during inflammation and is therefore relevant to examine the production of C1q by these cells and how this expression relates to the levels of expression of the receptor to C1q, namely gC1qR.

The monocyte adherence assay was conducted in the presence and absence of LPS, since it was curious to see if the expression of C1q and gC1qR would be affected by stimulation of the monocytes by LPS. C1q expression increased markedly in adherent monocytes during adherence, peaking at 72 hrs with a \log_{10} 3.5 fold differences in expression compared to 2hrs adherence (Figure 3.33). Incubation with LPS had a increased C1q expression. There was also an increase in gC1qR expression during adherence, with peaks observed at 48 and 72 hrs. In contrast to C1q, the presence of LPS downregulated the expression of gC1qR in comparison to without LPS. This was not surprising since gC1qR is not a ligand for LPS. These results confirm our previous data, showing enhancement of gC1qR surface expression on endothelial cells after 24 hr.

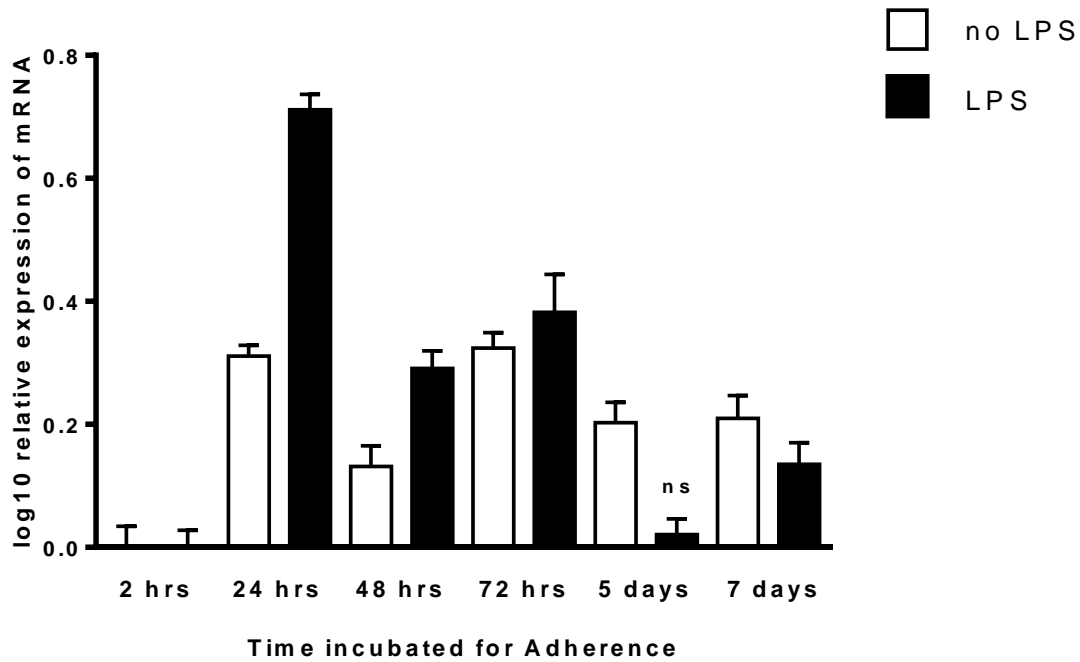


Figure 3.33. Expression of C1q by adherent human monocytes *in vitro*. The expression of C1q was measured using real time qPCR and the data normalised 18S rRNA gene expression as a control. Relative expression (RQ) calculated by using the 2hr timepoint as the calibrator. The RQ value was calculated using the formula: $RQ = 2^{-\Delta\Delta Ct}$. Assays were conducted twice in triplicate. Error bars represent \pm standard error of the mean. A two-side t-test was performed on the data, All samples showed significant expression compared to the calibrator ($p \leq 0.01$), except where noted: *: $0.01 < p < 0.05$; ns: not significant ($p \geq 0.05$). LPS , added to cultures at $20\text{ng}/\mu\text{l}$

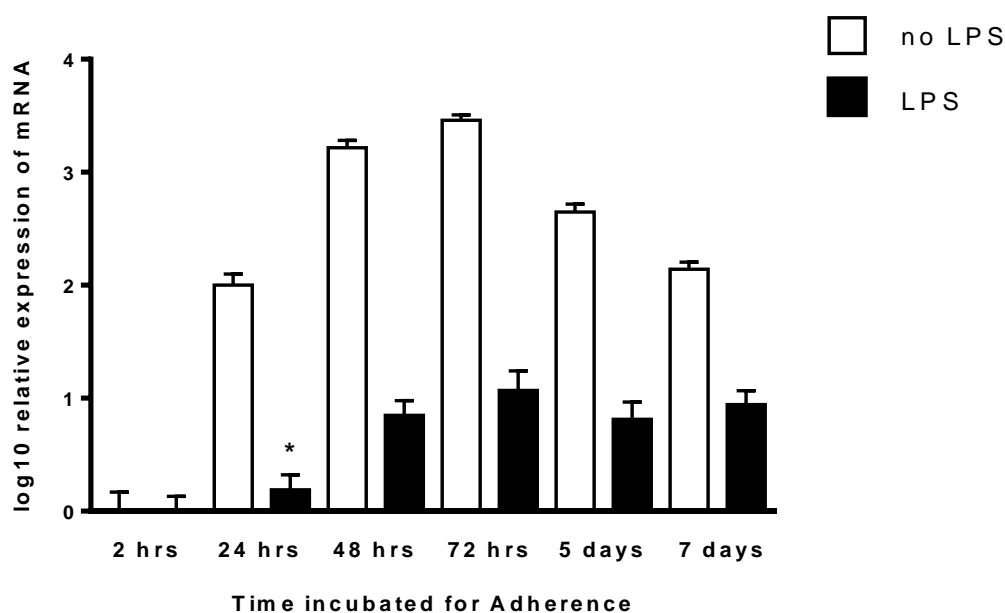


Figure 3.34. Expression of gC1qR by adherent human monocytes *in vitro*. The expression of gC1qR was measured using real time qPCR and the data normalised 18S rRNA gene expression as a control. Relative expression (RQ) calculated by using the 2hr timepoint as the calibrator. The RQ value was calculated using the formula: $RQ = 2^{-\Delta\Delta Ct}$. Assays were conducted twice in triplicate. Error bars represent \pm standard error of the mean. A two-side t-test was performed on the data.

3.5 Discussion

The heterotrimeric gC1q domain belonging to the C-terminal region of C1q contributes to the ligand binding property of C1q. C1q being a charge pattern recognition protein is able to bind to a range of ligands through its gC1q domain in the presence of calcium (Assimeh and Painter, 1975; Roumenia et al 2006). Intriguingly the configuration of the heterotrimeric gC1q module is also found in a range of non complement proteins (Ghai et al 2007; Carland and Gerwick, 2010, Tang et al., 2005) such as collagen VIII (Kishore et al 1999; 2000; 2004; Csako et al., 1981) precerebellin (Gerwick et al., 2000) and multimerin (Mei et al., 2008). Crystal structure analysis of the gC1q domain shows a sphere-shaped, heterotrimeric arrangement with the N and C terminal ends of each domain residing at the base of the trimer. Alongside this, the crystal structure has also identified an exposed Ca^{2+} ion located at the apex; this ion has been implicated to be important in the binding of gC1q to its ligand.

The main receptor on the cell surface identified for the gC1q domain is gC1qR. However the function and contributions of ghA, ghB and ghC in binding to gC1qR has not been specified. Therefore, recombinant forms of ghA, ghB, ghC and were expressed and purified in order to understand whether these C-terminal globular regions function individually or whether C1q's ability to bind its receptor is dependant on these globular head modules in a combined structure. In addition to the recombinant forms of ghA, ghB and ghC, single residue substitution mutants of each domain were also expressed and purified in an attempt to define the residues important in this interaction. Previous studies have shown that gC1qR inhibits complement activation by binding to the gC1q domain of C1q, thereby preventing IgG from binding to the globular heads. Therefore it was hypothesised that the binding sites for gC1qR and IgG may overlap, with literature showing that one of the IgG binding sites resides on Arg¹⁶² of the A chain. This has been made evident in the mutational studies where mutant R162E reduced the ability of C1q to bind to IgG by 35% (Kojouharova et al 2004). The role of arginine residues in the C1q globular domain has previously been considered important which is interesting as figure 3.26 suggests that the A chain is also preferential in the binding between C1q and gC1qR. Alongside this, previous experiments were carried out by Ghebrehiwet et al, 1997 that showed that the Arg residue Arg¹⁶² in C1q is significant in gC1qR binding. A peptide corresponding to the A-chain with the Arg residues at position 162 substituted to Glu showed no binding whereas the A-chain peptide with the Arg residues intact bound strongly to gC1qR. It has also been noted that residues 154-165 of the A chain is implicated in IgG binding (Marques et al., 1993) and inhibition studies have demonstrated that the complex of C1q and gC1qR prevented SRBCs to bind to C1q and diminished complement activation. This data supports the findings of our experiments as figure 3.28 clearly indicates that substitution of the Arginine residue in ghA abolishes its binding ability to gC1qR. , illustrating that within the A-chain, Arg 162 could be the most important region for this interaction.

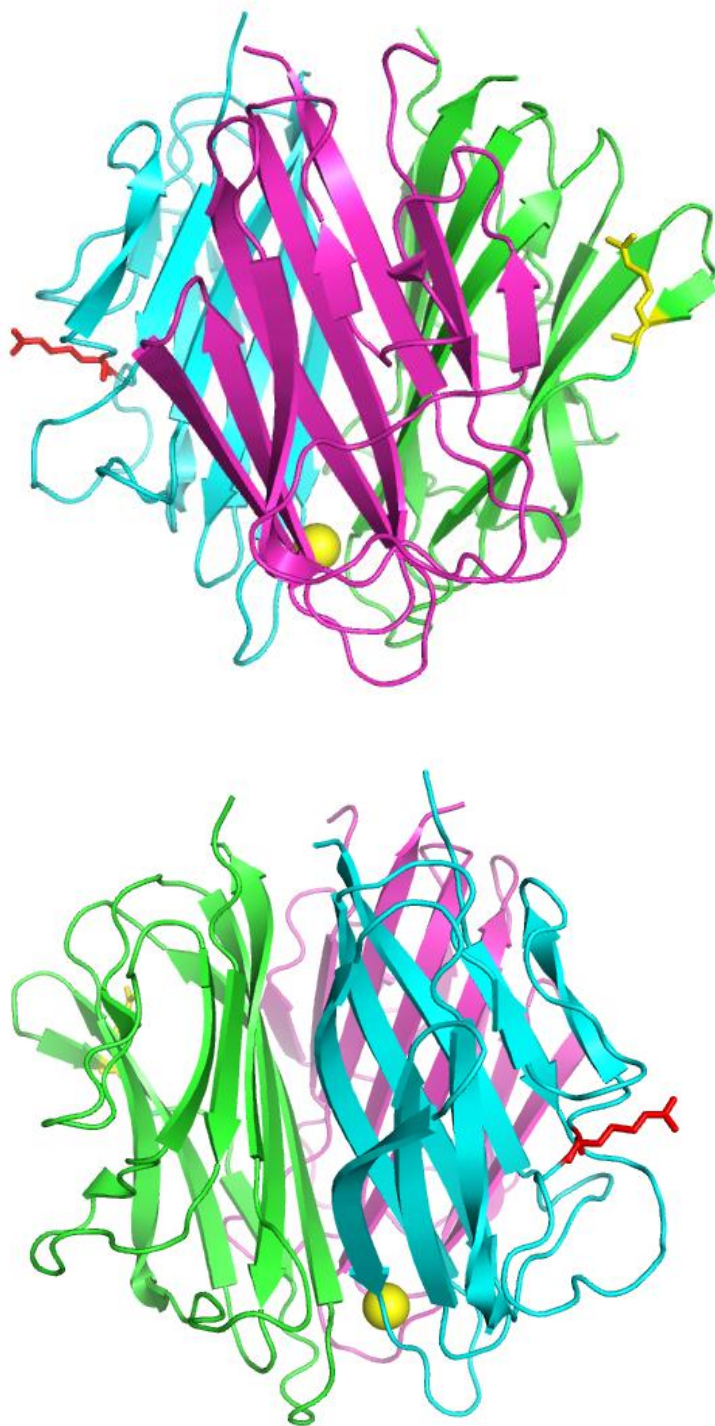


Figure 3.34: Structural analysis of gC1q showing mutated residues: a) Structure of gC1q globular head region, green, blue and magenta region represents ghA, ghB and ghC. Location of ghB mutant R114 is represented as a red stick situated at the side chain. ghA mutant R162 shown as a yellow stick. Calcium ion represented as a gold sphere. **b)** Reversed view of gC1q domain

We further examined the contributions of Arg¹¹⁴, Arg¹⁶³ and His¹¹⁷ (of ghB) and Arg¹⁵⁶ (of ghC) to the C1q-gC1qR interaction by substituting them with either neutral or negatively charged residues. Our experiments showed that the substitution of Arg to the negatively charged residue glutamine in amino acid 114 of the B chain increased binding to gC1qR even greater than the wild type ghB.

The observation that a substitution mutant is interacting better than the wild-type is of great interest. In addition, experiments involving chemical modification of certain amino acid residues have associated Arg¹¹⁴ of the B chain to be an important residue in IgG binding. This suggests that although the B chain requires ionic amino acids at this specific position for IgG binding, such strong ionic interactions situated in ghB are not as favourable for gC1qR binding, as glutamate at position 114 bound gC1qR with greater affinity than Arginine (Figure 3.28).

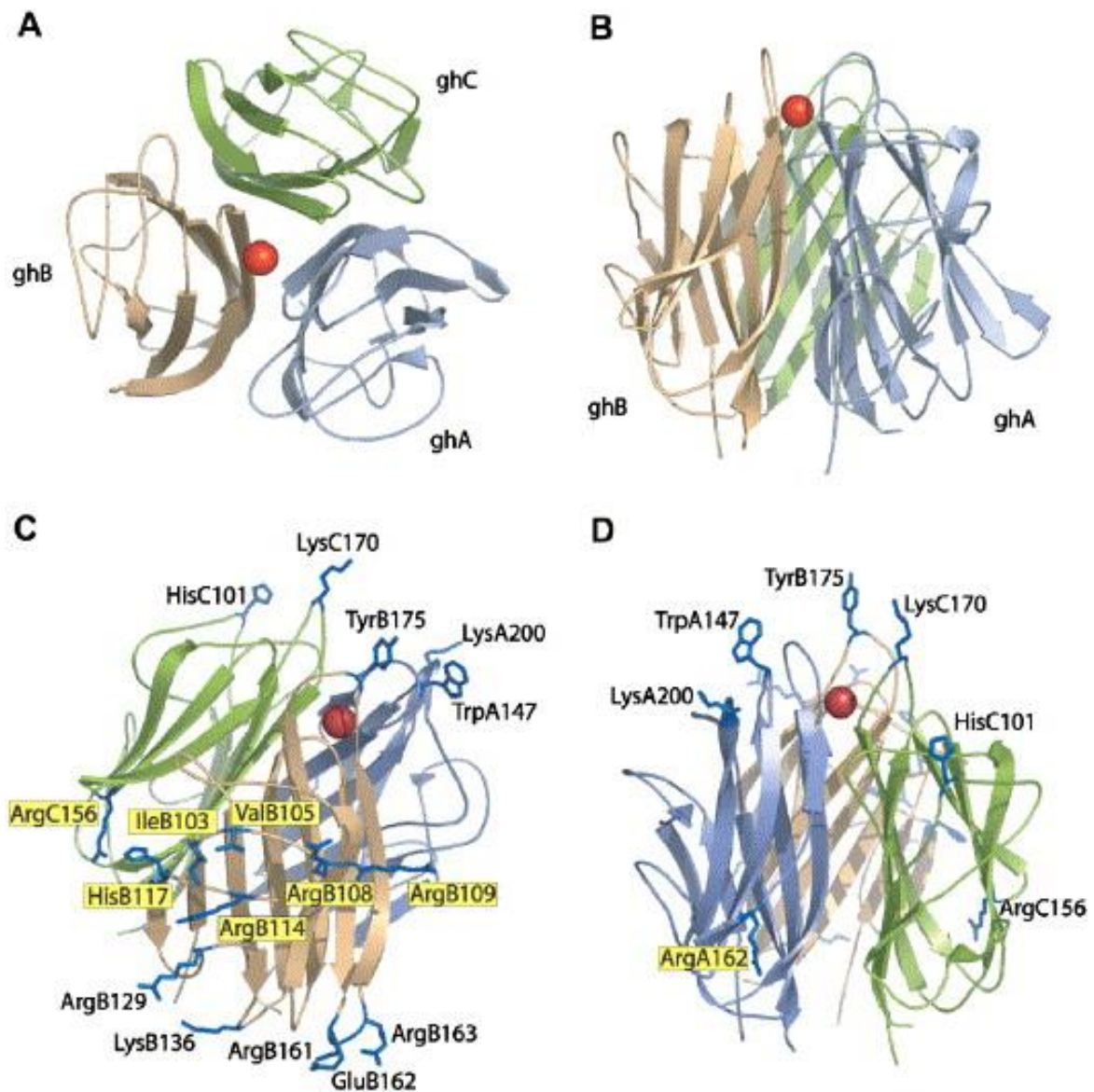


Figure 3.36: Crystal structure analysis of globular head region. A) Individual ghA, ghB and ghC heads showing situation of the Ca²⁺ ion at the apex. B) gC1q domain. C) Globular head domain showing location of important residues implicated in ligand binding found on ghA, ghB and ghC. D) gC1q domain illustrating vital residues of ghA, ghB and ghC.

The availability of a crystal structure of gC1qR (Jiang et al 1999) has enabled Ghebrehwet et al to generate gC1qR deletion mutants to confirm previous binding sites as well as identify new ones. The domains were deleted on the basis of holding a functional role. By analysing the interaction between the gC1qR deletion mutants and ghA, the preferential binder from C1q we can conclude that gC1qR holds additional binding sites for C1q. Residues 74-96 which have been noted as the C1q binding site on

gC1qR appeared to bind as strongly as WT gC1qR, whereas the HK binding site comprising of residues 204-218 and encoded by exon V demonstrated a drastic decrease in interacting with ghA. Deletion of residues 192-196 and 196-202 also displayed a reduction of binding to ghA implying that these residues are important for C1q binding. Remarkably, Ghebrehiwet et al 2011 has revealed that residues 192-196 and 196-202 are also important for HK binding as deletion of these residues abrogated the binding of gC1qR to HK. This therefore draws the conclusion that three binding sites have been revealed that appear to be important in interacting with both plasma proteins C1q and HK. HK has been associated with vascular permeability promoting angiogenesis (Guo and Coleman 2005). Therefore, the idea of C1q sharing an overlapping site with HK on gC1qR suggests that C1q could be used as a therapeutic molecule to intervene between gC1qR and HK binding to prevent vascular leakage and consequent inflammation.

The expression of C1q and gC1qR in adherent monocytes, using qPCR, appeared to suggest that the receptor and the effector protein i.e. C1q are up-regulated with adherence that mimics pro-inflammatory context. The concomitant expression was biphasic suggesting expression, first via preformed transcripts followed by new wave of transcription. These results appear to suggest that the two proteins are co-expressed under pro-inflammatory conditions and can regulate each other's functions. When LPS was used, the expression of C1q was upregulated, but gC1qR levels were reduced, suggesting that gC1qR is not directly involved in C1q-mediated effector functions involving LPS.

Chapter 4:

**Tripartite molecular interaction
between C1q, gC1qR and DC-SIGN:
Implications for HIV-1 pathogenesis**

4.1 Abstract

Dendritic Cells (DCs) are identified as the most potent antigen presenting cells within the immune system. Their ability to be the only APCs to prime naïve T-cells has become a hot topic of study due to the role of its receptor DC-SIGN, which in the last decade has been shown to regulate a wide range of immune functions. Recently this C-type lectin has emerged as a C1q binder, forming a trimeric complex involving gC1qR on the surface of immature DCs. We expressed and purified recombinant DC-SIGN and SIGN-R domains, the tetrameric form comprising of the entire extra cellular domain including the α helical neck, and the carbohydrate recognition domain in its monomeric form. Direct binding studies revealed that both variants were able to bind independently to the individual globular head modules ghA, ghB and ghC, with ghB being the preferential binder. Mutational analysis using single amino acid substitutions of the globular heads showed that Tyr^{B175} and Lys^{B136} appear critical in facilitating the C1q-DC-SIGN/SIGN-R interaction. Competitive studies also demonstrated that gC1qR and ghB share overlapping sites on DC-SIGN implying that HIV pathogenesis could be modulated due to the interplay of gC1qR-C1q with DC-SIGN.

4.2 Introduction

Dendritic Cells (DCs) are the most powerful antigen presenting cells (APCs) within the immune system (Dorfel et al., 2005) (Ni and O'Neill, 1997). They are capable of capturing and degrading pathogens by recognizing molecular patterns expressed by pathogens, through a series of pattern recognition receptors (PRR) known as C-type lectins (Weis et al., 1998) Essentially C-type lectins are recognized through their ability to bind carbohydrates in a calcium-dependent manner (Weis et al., 1992). The role of DCs within the immune system is vital in initiating immune responses due to being the only cell type able to activate naïve T-Cells (Austyn et al., 1983). This contact between DCs and T cells is vital in producing primary immune responses. Dendritic Cell specific Intracellular Adhesion Grabbing Non Integrin (DC-SIGN) is a C-type lectin expressed on DCs which, functions to mediate interactions between DCs and T cells through interacting with Intracellular Adhesion Molecule-3 (ICAM-3) (Geijtenbeek et al., 2000c). The importance of DC-SIGN as a mediator in this interaction has been supported by the inhibition of this binding preventing DC induced proliferation of resting T cells (Geijtenbeek et al., 2000c).

DC-SIGN has been shown to play a role in cell migration (Liu et al., 2002). DCs adhere to endothelial cells expressing high levels of ICAM-2 which bind DC-SIGN. Further interactions between Lymphocyte Function-Associated Antigen-1 (LFA-1) and ICAM-1 with ICAM-2 – DC-SIGN promote transendothelial migration of DCs, allowing them to travel from the blood to the lymphatic system where they can induce T cell responses. The role of DC-SIGN in boosting immunity has been further explored by Martinez et al who has shown that DC-SIGN stimulates CD3-activated T-cells to produce IL-2 which in turn enhances T- cell differentiation (Martinez et al., 2005). As well as participating in a range of functions to promote immunity, this receptor also engages in activities that bring upon immune suppression (Tong et al 2006) . This is evident through the ability of DC-SIGN to bind to a range of viral proteins and pathogens, including the glycolipid ManLAM of *Mycobacterium tuberculosis* (*M.tb*) (Turville et al., 2001). The binding of DC-SIGN to this cell wall component inhibits DC maturation through the suppression of Toll like Receptor (TLR-4) (Maeda et al., 2003). Such a cross-talk between TLRs and DC-SIGN that generates anti-inflammatory effects points out the two dimensional role of DC-SIGN in immunoregulation. DC-SIGN's immunosuppressive role is further highlighted

through its ability to bind to the HIV-1 envelope protein gp120 (Curtis et al., 1992), where instead of permitting entry and infecting DCs, this protein behaves as a reservoir by allowing DC to carry HIV-1 to the lymph nodes where it is transmitted to CD4⁺ T cells leading to immunodeficiency (Geijtenbeek et al., 2000b). The Hepatitis C Virus (HCV) envelope glycoprotein E2 is another viral protein DC-SIGN engages with (Pohlmann et al., 2003) to inhibit immunity. This is achieved through utilizing its high quality endocytic capability to internalize the viral antigen leading to the infection of DCs (Lozach et al., 2003). Structurally, DC-SIGN is composed of an extracellular domain which exists as a tetramer, stabilised by an α helical neck region, followed by a Carbohydrate Recognition Domain (CRD), (Mitchell et al., 2001). Its affinity for N-linked high mannose oligosaccharides is evident through its ligands gp120 and ICAM-3 being highly glycosylated, indicating that this binding is mediated through the CRD region (Curtis et al., 1992).

The importance of DC-SIGN as an immune molecule has been a central topic of study in the past decade. Possible therapeutic roles for DC-SIGN has been suggested owing to its ability to induce T cell proliferation when targeted with DC-SIGN antibodies conjugated with antigens (Singh et al., 2009). Sarkar et al 2013 have shown that the interaction between gp120 and DC-SIGN triggers a drop in IL-6 production by iDCs. In addition to this, gp120 binding to DC-SIGN has also been shown to suppress the anti-apoptotic activity of Nef and induce apoptosis in iDCs (Sarkar et al., 2013). Thus, HIV pathogenesis heavily relies on the interplay of molecular mechanisms involving DC-SIGN (Sarkar et al., 2013).

The interesting fact about DC-SIGN expression restricted to DCs points out the contradicting functions of this C-type lectin: its role as an immunostimulatory molecule facilitating DC interaction with naïve T cells to enhance immunity; and a promoter of HIV-1 infection via binding viral ligands. It has, therefore, become of great interest to discover possible modulators of HIV-1 infection that can interfere with such interactions. The targeting of glycans of glycoproteins using CBAs (carbohydrate binding agents) has become increasingly attractive area of research, indicating that such molecules could potentially act as inhibitors at preventing viral entry between the viral protein and its target cell (Balzarini and Van Damme, 2007). Such therapeutic strategies have proved to be effective at preventing DC-SIGN directed HIV-1 transmission to CD4⁺

T-cells (Balzarini et al., 2007). Very recently, the microbicide lectin drug GRFT (*Griffithsia*) isolated from the red algae, has been shown to not only inhibit DC-SIGN mediated capture and transmission of HIV-1 to CD4⁺ T-cells, but to also dissociate gp120 from the gp120-DC-SIGN complex (Hoorelbeke et al., 2013)

Recently, it has emerged that DC-SIGN binds to C1q via its globular head region (Hosszu et al., 2012), forming a trimeric complex with gC1qR, on the surface of immature DCs. Both C1q and gC1qR are known to associate with the viral envelope protein gp41 of HIV-1 (Fausther-Bovendo et al., 2010). C1q-gp41 has been shown to activate complement (Ebenbichler et al., 1991), whereas gC1qR has been identified with a novel role of being the receptor on CD4⁺ T-cells that binds to gp41 and targets healthy CD4⁺ T cells to Natural Killer (NK) cell-mediated lysis (Fausther-Bovendo et al., 2010). The recently discovered trimeric complex on immature DCs has been hypothesized to play a role in DC differentiation through signaling via DC-SIGN activating the NF κ B pathway. The ghA module has previously been shown to bind differentially to gp41 peptide (Kishore et al., 2003). Thus, we further examined the interaction between globular head modules and DC-SIGN, with the idea that this interaction may interfere with HIV-1 infection. Here, we show that ghB preferentially binds to DC-SIGN and SIGN-R. It also appears that ghB and IgG binding sites on DC-SIGN are overlapping and shared since Lys¹³⁶ and Tyr¹⁷⁵ on ghB, which have been previously shown to be significant for binding to IgG and vital for gC1q assembly (Gadjeva et al., 2010) are important for C1q-DC-SIGN binding.

Previously, structure-function studies have demonstrated that the CRD region of DC-SIGN is the specific site for ligand binding and only functions in the presence of the neck region within the Extra Cellular Domain (ECD) (Lozach et al., 2003). The use of recombinant forms of DC-SIGN tetramer and DC-SIGN monomer (CRD) alone has revealed the significance of the neck region in facilitating binding to its newly found ligand C1q.

4.3 Materials and methods

4.3.1 Construct expressing DC-SIGN Tetramer, DC-SIGN Monomer, SIGN-R Tetramer, SIGN-R Monomer

The cDNA was inserted in the pT5T expression vector at the *Bam*HI restriction site and the plasmids were used to transform *Escherichia coli* strain BL21/DE3.

4.3.2 Expression of DC-SIGN and SIGN-R as monomers and tetramers

Plasmids containing the full length and truncated cDNA of each protein were kindly provided by Dr. Daniel Mitchell from the University of Warwick. The cDNA was inserted into the pT5T expression vector at the *Bam*HI restriction site and the plasmids were transformed in *Escherichia coli* strain BL21/DE3 (Mitchell et al, 2002) The purified plasmid DNA was transformed into BL21 DE3 cells using the protocol described in Material and Methods chapter. Various constructs designed to express DC-SIGN tetramer, DC-SIGN monomer, SIGN-R tetramer and SIGN-R monomer containing transformed cells were plated onto agar with ampicillin added, whereas SIGN-R monomer transformant was plated onto agar with kanamycin added. Both antibiotics were added to a concentration of 50µg/ml and incubated at 37°C overnight. 150µl of cells were added to each plate and spread using a sterile plastic spreader. Protein expression was performed using Luria-Bertani medium with 50mg/ml of ampicillin. A single transformed colony of DC-SIGN or SIGN-R was grown overnight at 37°C in 15ml LB and diluted in 1 litre of LB that was incubated at 37°C until an A550 of 0.7 was reached. The culture was induced with 10mM isopropyl-b-D-thiogalactoside (IPTG) and was incubated for an additional 3 hours at 37°C. Bacteria were centrifuged at 4500rpm for 15 minutes at 4°C and the pellet was collected.

4.3.4 Inclusion body preparation

The cell pellet was treated with 22ml of lysis buffer (100mM Tris pH 7.5, 0.5M NaCl, lysozyme, 2.5mM EDTA and PMSF) and left to shake for 1 hour at 4°C. Cells were sonicated on ice for 10 cycles for 30 seconds with 2 minute gaps to ensure complete lysis of cells. The lysate was centrifuged at 10,000rpm for 15min at 4°C. The supernatant was then discarded and the inclusion body pellet was mixed with 20ml of

6M Urea, 100mM Tris pH 7.5 and 20 μ l β -mercapthethanol. This solution was left shaking on a rotor in the cold room for 1 hour

4.3.5 Refolding protocol

The mixture was then centrifuged at 13,000rpm for 30mins at 4°C. . The supernatant was collected into a beaker and whilst gently placed on a magnetic stirrer 80ml of loading buffer (1M NaCl, 25mM Tris pH 8.0, 5mM CaCl₂) was added slowly drop by drop using a drop wise diluting method to create a gradient. The mixture was then dialysed against two litres of loading buffer and changed three times every six hours.

4.3.6 Purification of recombinant proteins

Following further centrifugation at 13,000 rpm for 15mins at 4°C, the supernatant was loaded onto a mannon agarose column (5ml) pre-equilibrated 5 times with loading buffer. The column was washed again with 5 bed volumes of loading buffer and purified protein was collected in 1ml fractions of elution buffer (1M NaCl, 25mM Tris-HCL pH 7.8, and 2.5mM EDTA). The absorbance was read at 280nm and fractions were frozen at -20°C. Purity of protein was analysed by SDS-PAGE.

4.3.7 Direct Binding ELISA

Microtitre wells were coated in duplicates with different concentrations of protein (DC-SIGN or SIGN-R) (5, 2.5, 1.25 and 0.625 μ g/well in bicarbonate buffer pH 9.6) and left overnight at 4°C. Wells were blocked with 100 μ l of 2%v/w BSA in PBS for for 2 hours at 37°C. Following three washes with PBS + 0.05% Tween, 2.5 μ g/well of ghA, ghB and ghC (100 μ l) was added in calcium buffer (50mM Nacl, 100mM Tris and 5mM CaCl₂) MBP was used as a negative control. The plate was incubated at 37°C for 1.5hours and a further 1.5hours at 4°C. The wells were washed and the bound protein was detected with anti-MBP mAb in PBS (1:5000), followed by rabbit anti-mouse IgG-HRP (1:5000). Colour was developed using OPD and read at 415nm

4.3.8 Competitive ELISA

The optimum concentration for DC-SIGN and SIGN-R binding was chosen based on the result of the direct binding ELISA. Proteins were immobilized on microtitre wells by overnight incubation at 4°C of 100 μ l of 5 μ g/well in carbonate/bicarbonate buffer, pH

9.6. Wells were blocked with 2%v/w BSA in PBS for 2 hours at 37°C. Following washing with PBS + 0.05% Tween, the plate was incubated with a constant concentration (5µg/well) of the first competing protein (gC1qR) and various concentrations (5, 2.5, 1.25, 0.625µg/well) of the second competing protein (ghB) in calcium buffer to give a total of 100µl per well. After incubating for 1.5 hours at 37°C and 1.5 hours at 4° the microtitre wells were washed and rabbit anti-gC1qR pAb (1:000) in PBS was added and incubated for a further 1 hour at 37°C. Bound protein was detected by Protein A-HRP conjugate (1:5000) and developed using OPD. Data was plotted to determine inhibitory values of competitive ligand binding.

4.3.9 Western Blotting

ghA, ghB and ghC (15µg) were separated by 12% SDS-PAGE gel and transferred onto PDVF membrane for 1 hour at 320ma. Membrane was blocked in 5% non fat milk (1 hour at room temperature) and 20µg of DC-SIGN in loading buffer was added and incubated (overnight at room temperature). The blot was washed three times for 10 minutes in PBS containing 0.05% Tween and incubated in primary antibody pAb anti-DC-SIGN (1:1000) in 1% non fat milk (2 hours at 37°C). Following subsequent washes, the membrane was incubated with Protein A conjugated HRP (1:1000) (1 hour at room temperature). The blot was developed using DAB substrate

4.4 Results

4.4.1 Expression and Purification of DC-SIGN and SIGN-R tetramer and monomer

Both DC-SIGN and SIGN-R comprising of the entire extracellular domain and the CRD region alone were expressed in *E. coli* and purified on Mannose Sepharose. The CRD fragments of DC-SIGN and SIGN-R bound mannose weakly as they eluted in the wash fraction. The ECD domains of both DC-SIGN and SIGN-R bound to mannose with much greater affinity in the presence of Ca^{2+} and eluted with EDTA

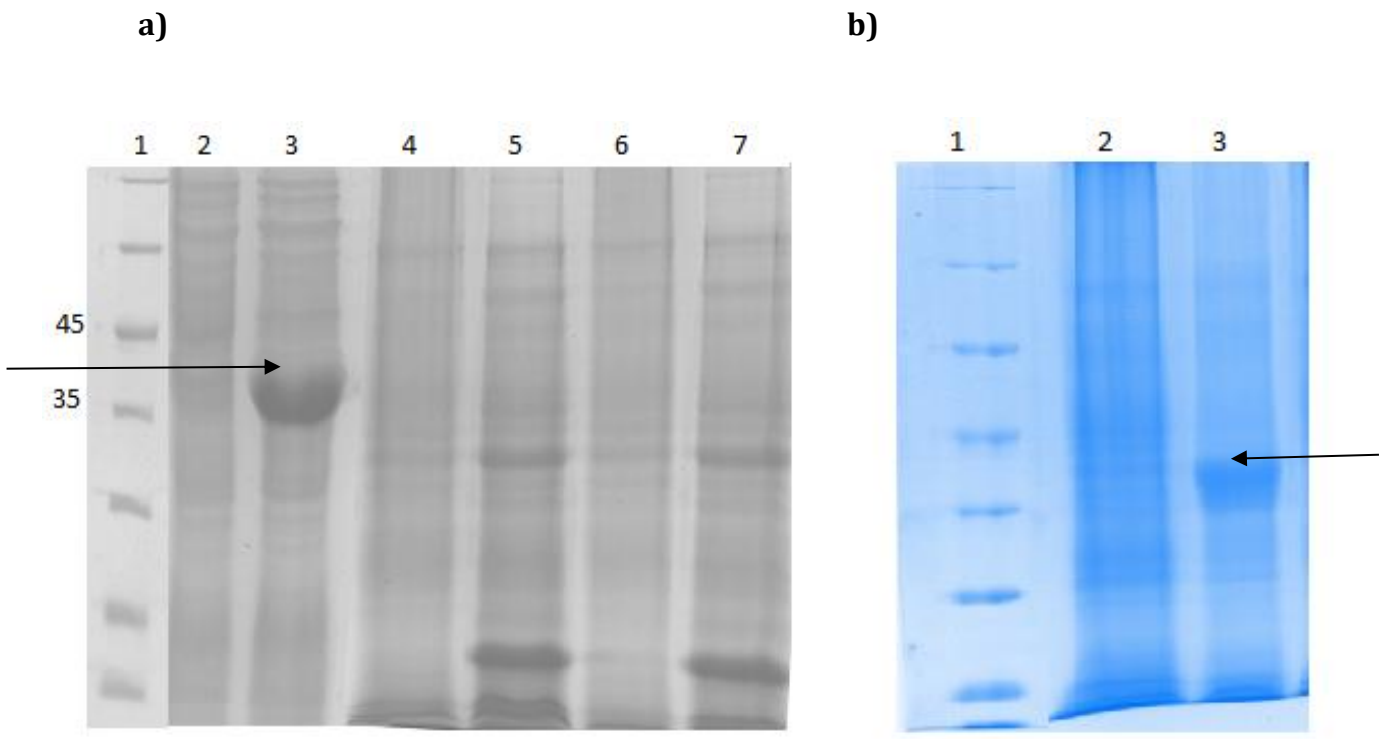


Figure 4.1: Induction of DC-SIGN Tetramer, DC-SIGN Monomer, SIGN-R Tetramer, SIGN-R Monomer: a) Lane 1: Molecular Marker, Lane 2: Uninduced DC-SIGN tetramer, Lane 3: Induced DC-SIGN tetramer, Lane 4: Uninduced DC-SIGN monomer, Lane 5: Induced DC-SIGN monomer, Lane 6: Uninduced SIGN-R Monomer, Lane 7: Induced SIGN-R Monomer. **b)** Lane 1: Molecular marker, Lane 2: Uninduced SIGN-R tetramer, Lane 3: Induced SIGN-R Tetramer

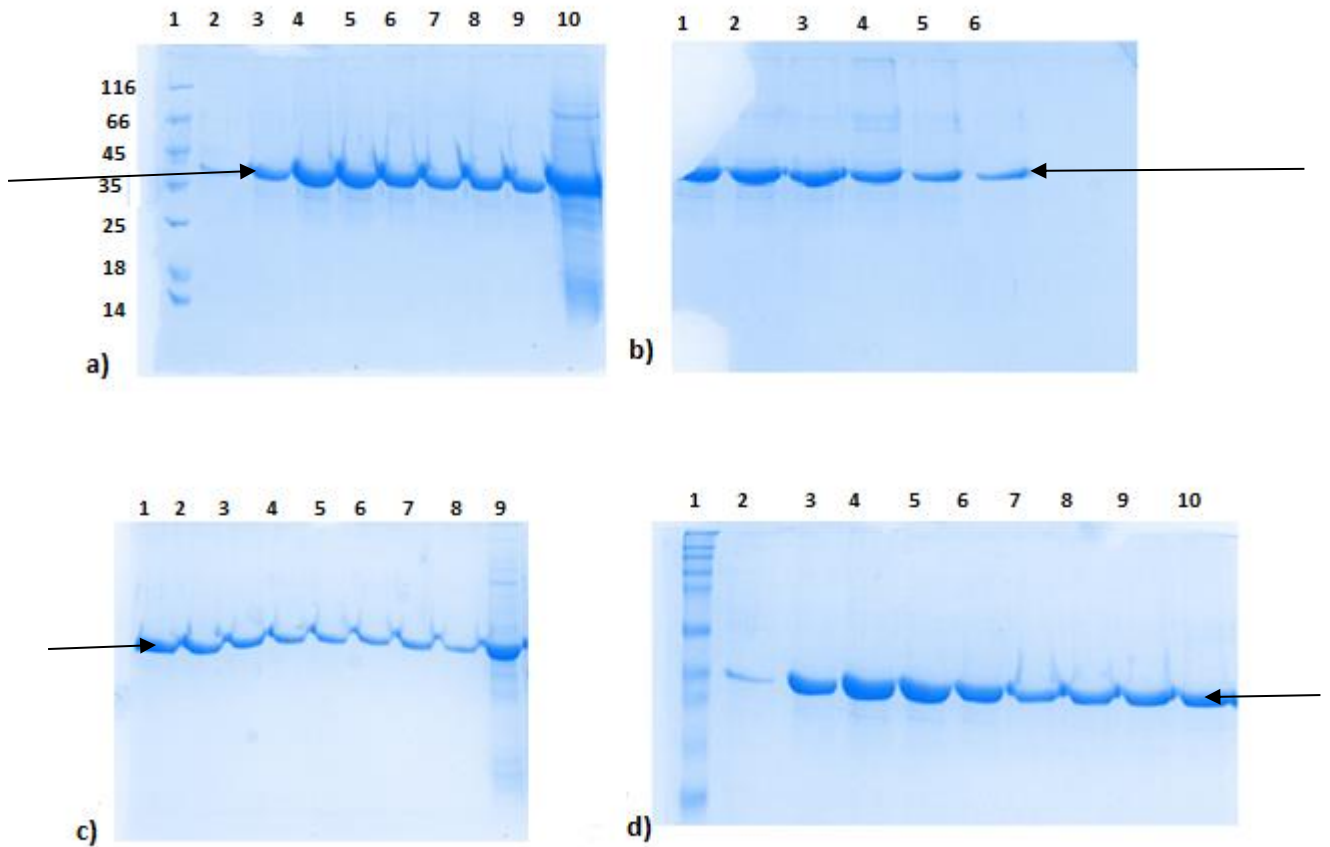


Figure 4.2: SDS-polyacrylamide gel electrophoresis of purified soluble a) and b) DC-SIGN Tetramer, c) and d) SIGN-R Tetramer purification by Mannon -Agarose affinity chromatography. a) Lane 1: Molecular marker, Lane 2-9: purified DC-SIGN tetramer, Lane 10: Flow through. b) Lane 1-6: Purified DC-SIGN tetramer. c) Lane 1-9: Purified SIGN-R tetramer. d) Lane 1: Molecular marker, Lane 2-10: Purified SIGN-R tetramer

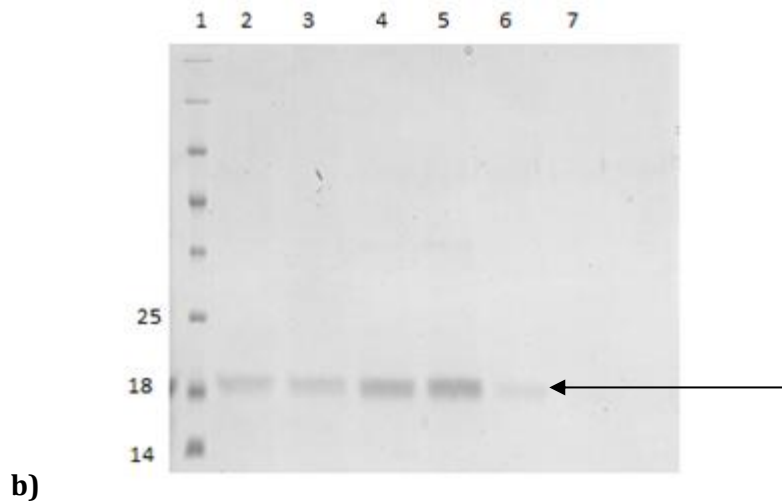
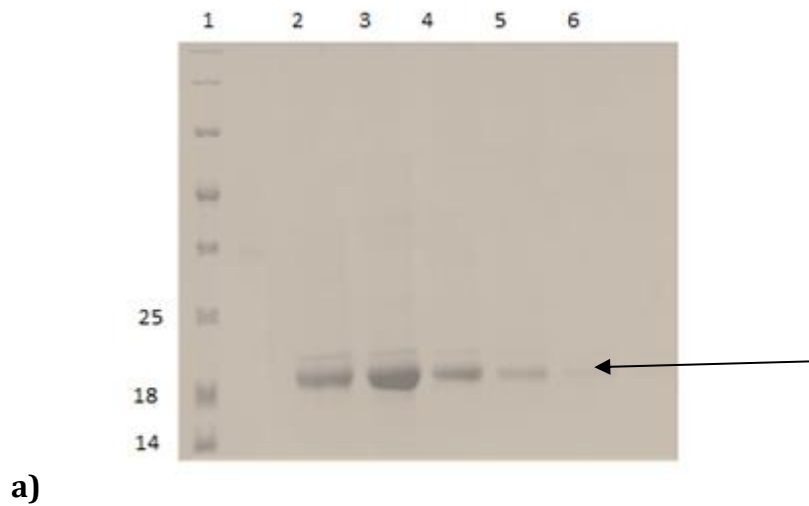


Figure 4.3: SDS-polyacrylamide gel electrophoresis of purified soluble a) DC-SIGN Monomer, b) SIGN-R Monomer purification by Mannon -Agarose affinity chromatography. Proteins were expressed in 1 litre of bacterial culture and elution fractions were collected in 1ml fractions on a 5ml column in buffer containing 5mM EDTA. 12% gel was stained with Coomassie Blue. A) *Lane 1: Molecular Marker, Lane 2-6: Eluted purified DC-SIGN Monomer . B) Lane 1: Molecular Marker, lane 2-6: Eluted purified SIGN-R Tetramer.* Expression is evident at 18kDa

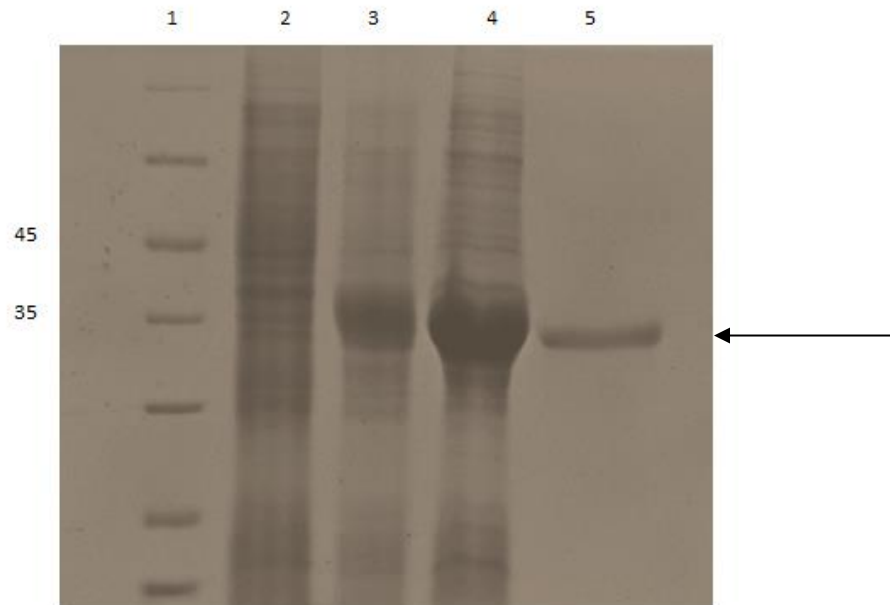


Figure 4.4: Expression and purification of DC-SIGN R: *Lane 1:* Molecular marker , *Lane 2:* Uninduced sample, *Lane 3:* Induced sample, *Lane 4:* Supernatant after sonication, *Lane 5:* Purified sample after elution. Expression is evident at 40kda

4.4.2 Both DC-SIGN and SIGN-R Tetramer and Monomer bind to C1q

Previously, Kang et al has shown that SIGN-R interacts with C1q and recently, work by Hosszu et al revealed that DC-SIGN bound directly to C1q. In this present study we sought to determine whether this binding is mediated solely through the CRD region, or whether the alpha helical neck is also a necessity. We examined direct binding of both the tetrameric and monomeric variants of DC-SIGN and SIGN-R with purified human C1q on microtitre plates. Figures 4.5 and 4.6 shows the results of the ELISA represented that both DC-SIGN and SIGN-R in their full length configuration (Tetramers) and the CRD alone (monomers) were able to bind to C1q in a dose dependent manner.

Experiment showed a strong binding of the tetramers to C1q when compared to the CRD region alone, with the ability of C1q to bind nearly 50% more when the alpha helical neck is intact. Furthermore the results above also demonstrate that C1q has a greater affinity to SIGN-R than DC-SIGN.

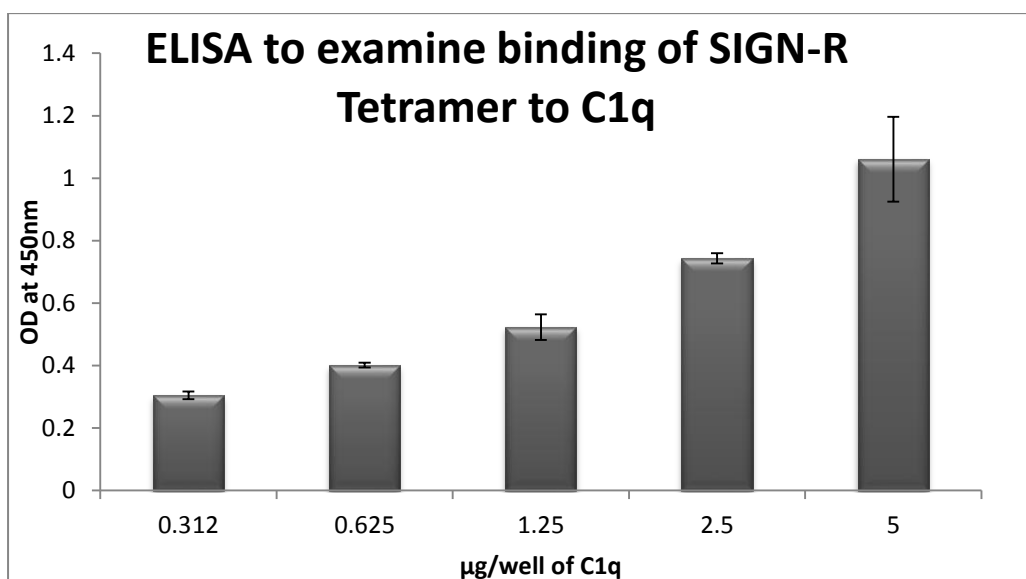
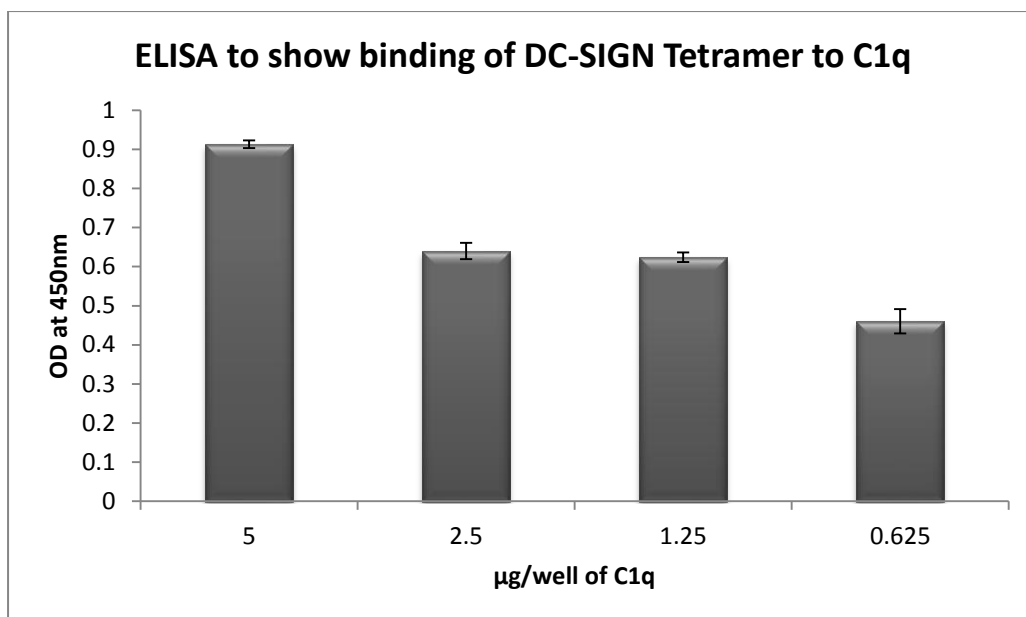


Figure 4.5: ELISA to show binding of C1q to DC-SIGN and SIGN-R: C1q was coated at different concentrations (5, 2.5, 1.25, 0.625µg/well) and left overnight at +4. Contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37°C. The wells were washed 3 times with PBS + 0.05% Tween and 2µg/well of DC-SIGN Tetramer or SIGN-R Tetramer was added in Calcium buffer (5mM CaCl₂) and incubated at 1.5 hours at 37 degrees and 1.5 hours at 4°C. Bound protein was probed with anti-DC-SIGN pAb (1:1000) or anti SIGN-R mAb (1:500) following Protein A HRP (1:1000) and detected using OPD.

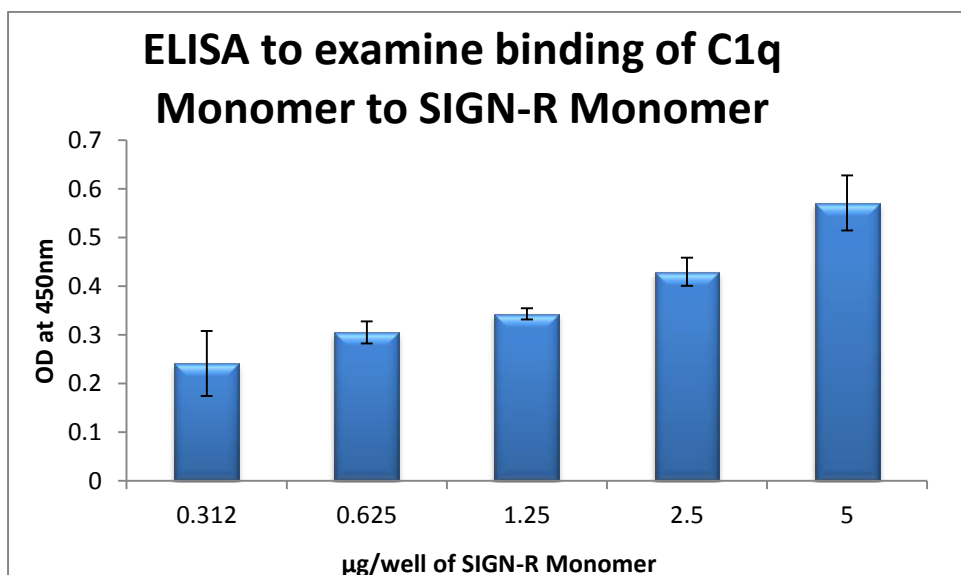
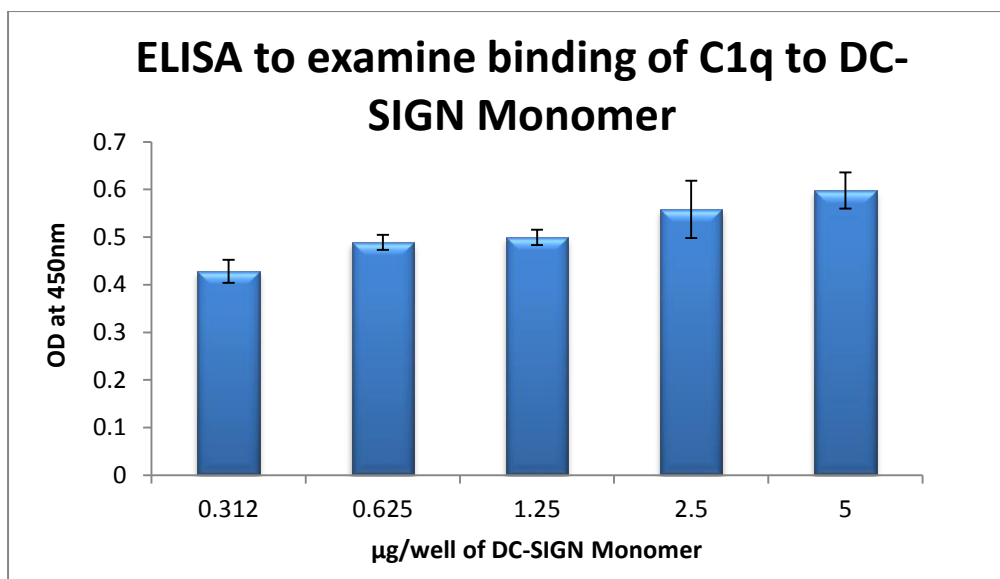


Figure 4.6: ELISA to show binding of C1q to DC-SIGN Monomer and SIGN-R Monomer: DC-SIGN Monomer and SIGN-R monomer were coated at different concentrations (5, 2.5, 1.25, 0.625µg/well) and left overnight at 4°C. Contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37°C. The wells were washed 3 times with PBS + 0.05% Tween and 2µg/well of C1q was added in Calcium buffer (5mM CaCl₂) and incubated at 1.5 hours at 37 degrees and 1.5 hours at +4. Bound C1q was probed with anti-C1q pAb (1:1000) following Protein A HRP (1:1000) and detected using OPD.

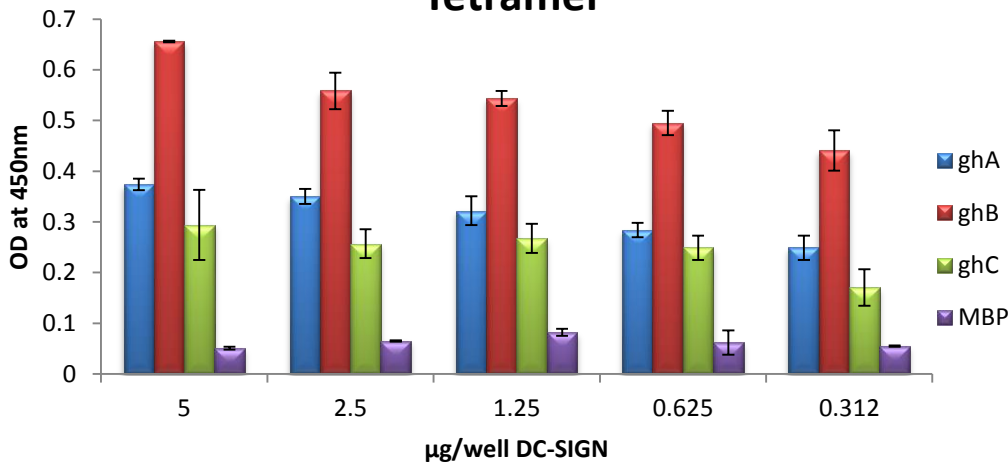
4.4.3 DC-SIGN and SIGN-R neck region is required for binding to C1q individual globular chains

Fragments of DC-SIGN and SIGN-R comprising of the complete extracellular domain and the CRD region were expressed in *E.coli* and purified on mannan-agarose column. Ligand binding studies were performed by ELISA where tetrameric and monomeric DC-SIGN and SIGN-R coated on microtitre wells were probed with ghA, ghB and ghC to examine whether these globular heads are able to bind to the ECD and CRD with similar avidity. Our experiments show that ghA, ghB and ghC bound with much greater affinity to DC-SIGN and SIGN-R Tetramer in comparison to the monomeric CRD variants, indicating that the neck is also preferentially required for the individual globular heads to bind efficiently. Figures 4.7 and 4.8 below also reveal that ghA, ghB and ghC bind SIGN-R with greater affinity than DC-SIGN.

4.4.4 DC-SIGN and SIGN-R bind preferentially to ghB

C1q binds to DC-SIGN via its globular head region, and due to the individual modularity within the gC1q region, we sought to map which chain is specific for DC-SIGN binding. By using single monomeric fragments of individual globular head chains ghA, ghB and ghC, we were able to assess which region of the C1q globular head is specific to DC-SIGN and SIGN-R. When DC-SIGN and SIGN-R were coated on microtitre wells and probed with ghA, ghB and ghC, all three globular heads bound DC-SIGN and SIGN-R in a dose dependent manner indicating that all three heads are capable of binding to the ligands independently. Furthermore, our experiments show that it is the ghB chain of C1q that is preferential in this binding to DC-SIGN as the ghB module bound much better to DC-SIGN when compared to ghA and ghC. In addition, ghB was the better binder to SIGN-R. Interestingly ghA was able to bind better with SIGN-R than DC-SIGN (Figure 4.7 and 4.8). Direct binding ELISA showed that both DC-SIGN and SIGN-R had less affinity for ghC when compared with ghA and ghB (figures 4.7 and 4.8). The binding properties of ghA, ghB and ghC for DC-SIGN and SIGN-R Tetramer were similar when the CRD region was probed with the individual globular heads. Although the binding of ghA, ghB and ghC to the CRD domain was significantly lower, ghB was still the most important chain in its interaction with DC-SIGN and SIGN-R monomer (figure 4.7b and 4.8b).

Binding of ghA, ghB and ghC to DC-SIGN Tetramer



Binding of ghA, ghB and ghC to DC-SIGN Monomer

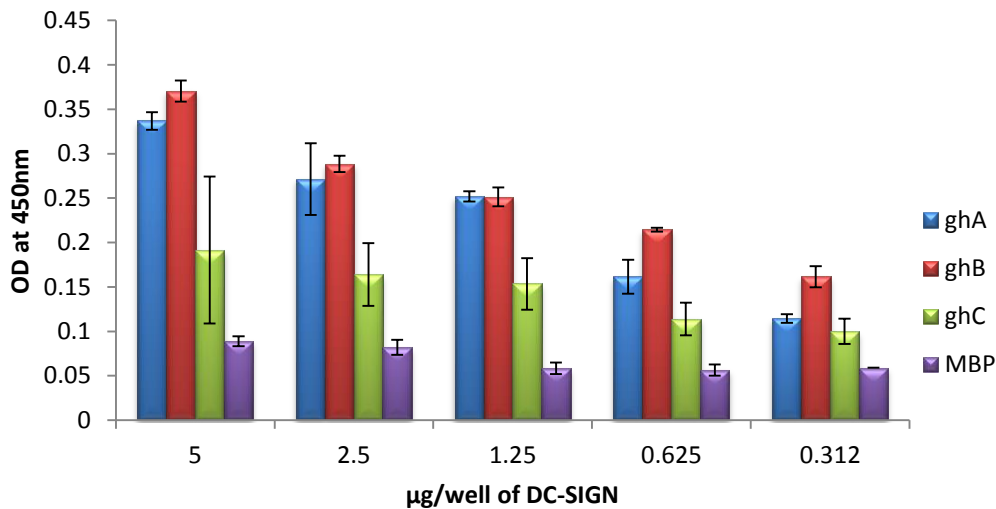


Figure 4.7: Binding of ghA, ghB and ghC to (a) DC-SIGN Tetramer and (b) DC-SIGN Monomer: Different concentrations of DC-SIGN Tetramer (a) and DC-SIGN Monomer (b) (5, 2.5, 1.25µg/well) was coated on microtitre wells in carbonate buffer and incubate overnight at 4°C. Wells were blocked in 2% BSA in PBS, following washing. ghA, ghB, ghC and MBP (2.5µg/well) were added and incubated. Wells were washed again and bound protein was detected using mAb MBP following IgG-HRP.

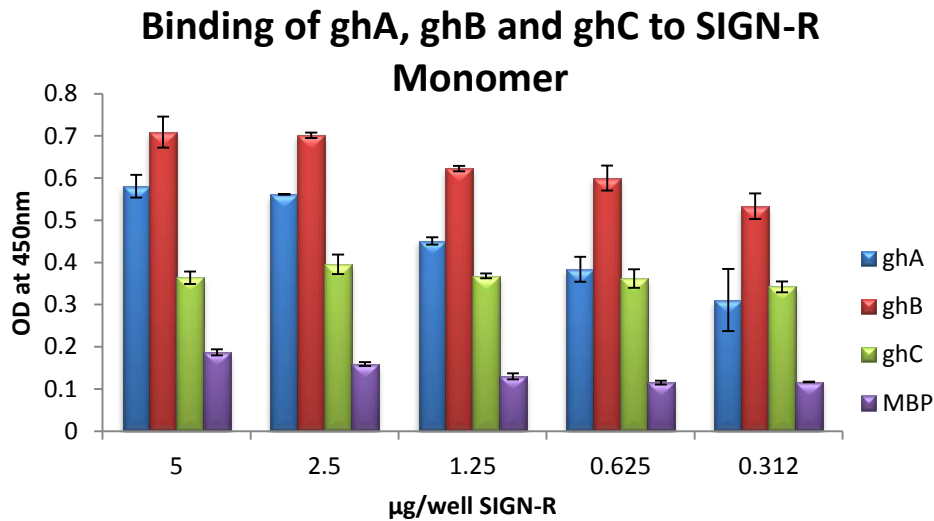
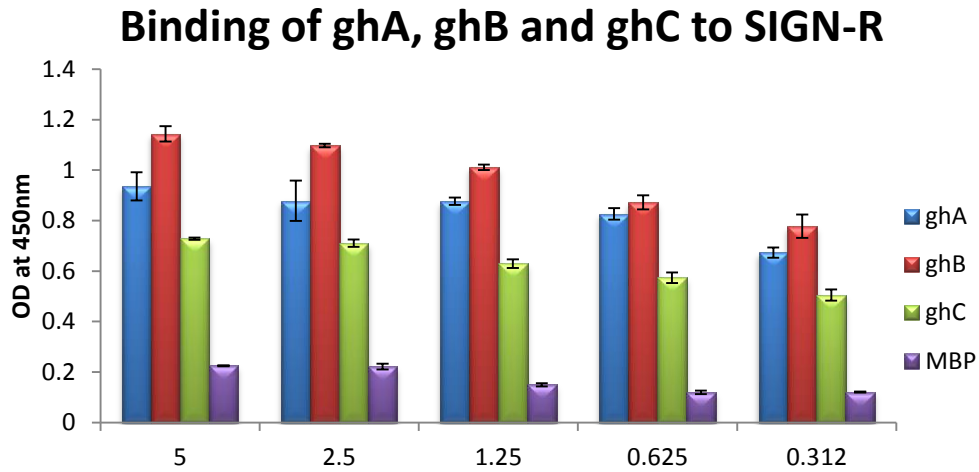


Figure 4.8: Binding of ghA, ghB and ghC to (a) SIGN-R Tetramer and (b) SIGN-R Monomer: Different concentrations of SIGN-R Tetramer (a) and SIGN-R Monomer (b) (5, 2.5, 1.25µg/well) was coated on microtitre wells in carbonate buffer and incubated overnight at 4 degrees. Wells were blocked in 2% BSA in PBS for 2 hours at 37 degrees. Following this, wells were washed 3 times in PBS + 0.05% Tween and ghA, ghB, ghC and MBP (2.5µg/well) were added to each well and the plate was incubated at 37 degrees for 1.5 hours and at 4 degrees for 1.5 hours. Wells were washed again and bound protein was detected using mAb MBP following IgG-HRP.

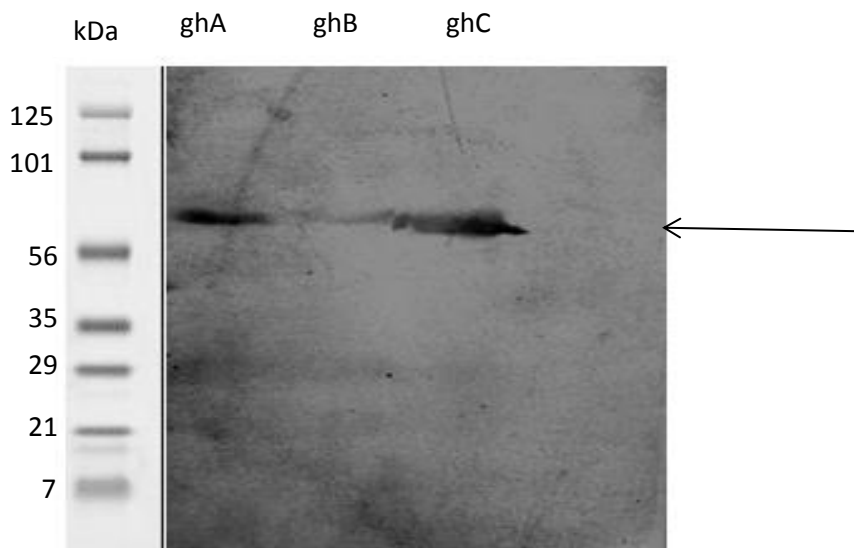


Figure 4.9: Far western blot of ghA, ghB and ghC with DC-SIGN: 15 μ g of ghA, ghB and ghC was loaded on a gel in three wells and run. The gel was transferred onto PDV membrane for 1 hour. The membrane was then blocked with 5% non-fat milk in PBS for 2 hours. 50 μ g of DC-SIGN in PBS was added to the membrane and incubated overnight at room temperature. The membrane was then washed 3 times in PBS-Tween 0.02% for 15 minutes each and 10 μ l of mAb anti-MBP in 2% non fat milk was added and incubated for 2 hours at 37 degrees. Following repeated washing, 10 μ l of IgG-HRP in 2% non fat milk was added and incubated for 1 hour at room temperature. The bands were developed using DAB tablets dissolved in water.

4.4.5 Examining the ability of DC-SIGN and SIGN-R to bind to the ghA substitution mutants

The ability of R162E and R162A to bind to gC1qR was assessed by ELISA. Both substitution mutants bound DC-SIGN and SIGN-R in a dose dependent manner (figure 4.10 and 4.12). It appeared that SIGN-R was able to interact with the ghA substitution mutant R162E nearly as efficiently as it did with Wild Type ghA (Figure 4.13) showing a reduction in binding of only 15%. R162A, on the other hand, bound SIGN-R with much less affinity showing a drop of 33% (figure 4.13). Considering DC-SIGN and SIGN-R are both highly conserved, R162E was able to interact with DC-SIGN reasonably weaker than it did with SIGN-R (figure 4.11), showing a 40% reduced binding as opposed to 15% (seen with SIGN-R.) The mutant R162A bound DC-SIGN in a similar manner as it did to its homologue SIGN-R showing a reduced binding of 30% (figure 4.11).

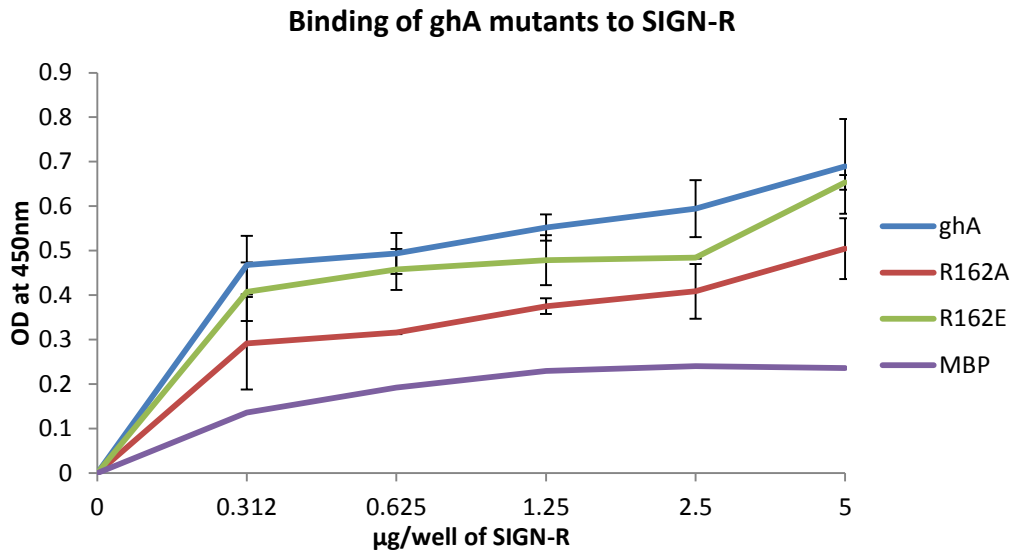


Figure 4.10: Binding of ghA, ghA-R162E and R162E to SIGN-R Tetramer: Different concentrations of SIGN-R tetramer (5, 2.5, 1.25µg/well) were coated on microtitre wells in carbonate buffer and incubated overnight at 4 degrees. Wells were blocked in 2% BSA In PBS for 2 hours at 37 degrees. Followig this, wells were washed 3 times in PBS + 0.05% Tween and ghA, ghA-R162A, ghA-R162E and MBP (2.5µg/well) were added to each well and the plate was incubated at 37 degrees for 1.5 hours and at 4 degrees for 1.5 hours. Wells Were washed again and bound protein was detected using mAb MBP following IgG-HRP.

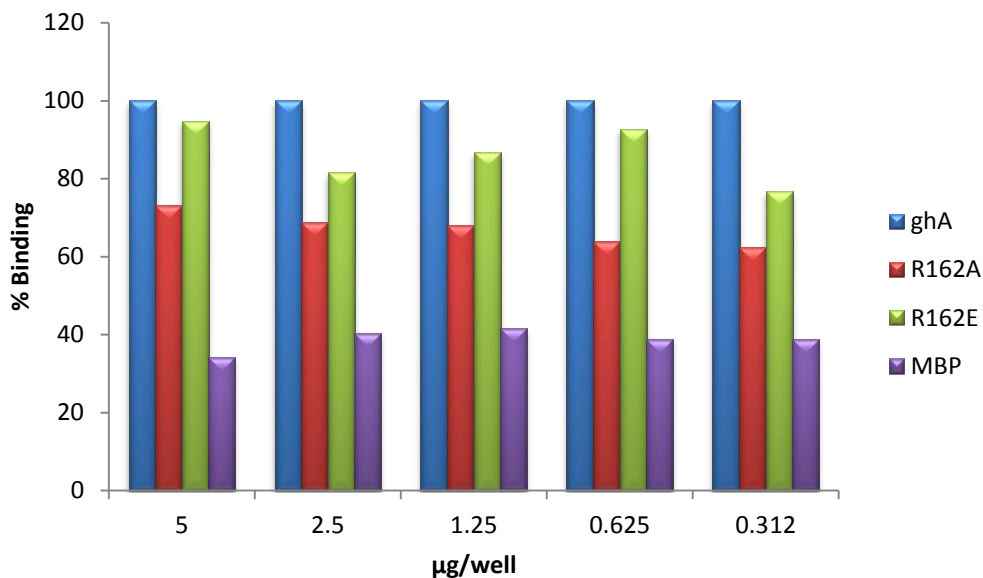


Figure 4.11: Percentage binding of ghA mutants to SIGN-R: To qualitatively asses binding inhibitory properties of the ghA mutants R162A and R162E, the percentage binding was worked out by using WT ghA as a 100% positive control

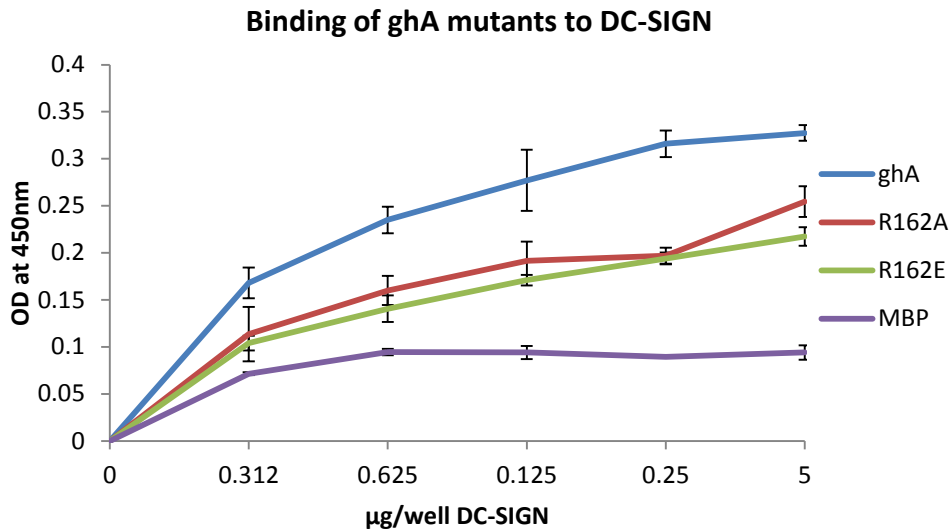


Figure 4.12: Binding of ghA, ghA-R162E and R162E to DC-SIGN Tetramer: Different concentrations of DC-SIGN tetramer (5, 2.5, 1.25µg/well) were coated on microtitre wells in carbonate buffer and incubated overnight at 4 degrees. Wells were blocked in 2% BSA In PBS for 2 hours at 37 degrees. Following this, wells were washed 3 times in PBS + 0.05% Tween and ghA, ghA-R162A, ghA-R162E and MBP (2.5µg/well) were added to each well and the plate was incubated at 37 degrees for 1.5 hours and at 4 degrees for 1.5 hours. Wells Were washed again and bound protein was detected using mAb MBP following IgG-HRP.

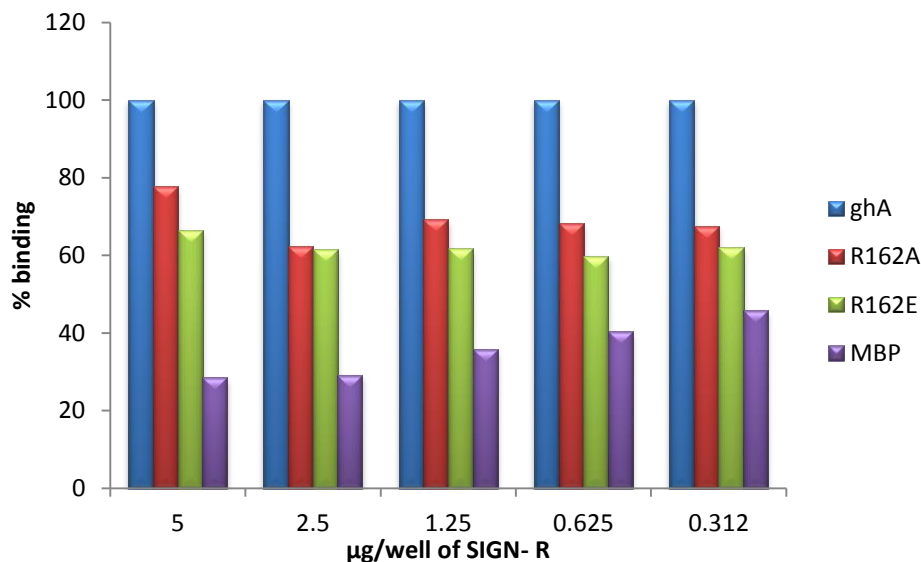


Figure 4.13: Percentage binding of ghA mutants to SIGN-R: To qualitatively assess binding inhibitory properties of the ghA mutants R162A and R162E, the percentage binding was worked out by using WT ghA as a 100% positive control

4.4.6 The contributions of ghB substitution mutants to DC-SIGN and SIGN-R binding

Using ELISA, we examined the ability of DC-SIGN to bind to the ghB substitution mutants R114Q, R114Q, R163E, R163A, R129A, R129E, H117D, T175L and L136G (Kojouharova et al 2004). Results showed that all the ghB substitution mutants bound both DC-SIGN and SIGN-R in a dose dependent manner (figure 4.14 and 4.16). Substituting Arg¹¹⁴ to Gln and Ala resulted in a reduction of 50% in DC-SIGN (figure 4.15) and SIGN-R binding (figure 4.17), suggesting that the Arg residue at this point plays an important role in the C1q-DC-SIGN/SIGN-R interaction.

Substituting the ghB mutant Arg¹²⁹ with Glu and Ala caused a small reduction of ~20% binding with DC-SIGN (figure 4.15) with these mutants exhibiting similar binding ability to SIGN-R (10-25%) (figure 4.17). When Arg¹⁶³ was replaced with the negatively charged Glu, its affinity for DC-SIGN and SIGN-R was reduced to 40% (figure 4.15 and 4.17), whereas substitution with Ala resulted in 30% reduction for DC-SIGN (figure 4.15) and 20% for SIGN-R (figure 4.17). A greater reduction in DC-SIGN binding of 60% was observed for the ghB mutant His¹¹⁷ substituted for Asp (figure 4.15), whereas the ability of H117D to interact with SIGN-R was knocked down by 30% (figure 4.17). For the ghB module the Tyr¹⁷⁵ substitution to Leu had the most significant effect showing a dramatic decrease of 70-75% in binding to both DC-SIGN and SIGN-R (figure 4.15 and 4.17). Similarly, L136G bound less to DC-SIGN showing a drop of 50-60% (figure 4.15).

4.4.7 Residue L136 and T175 of the ghB module is important for IgG binding is also involved in DC-SIGN and SIGN-R binding

Given that DC-SIGN binds to ghB (this study) as well as that DC-SIGN binds to C1q at the IgG binding site (Hosszu et al, 2012) we sought to identify the complementary binding sites. Via a series of ghB substitution mutants (Kojouharova et al., 2004), the interaction between the ghB chain of C1q and DC-SIGN revealed two important substitution mutants; T175L and L136G (Figure 4.14 and 4.15). As reported previously, L136 and T175 residues are important in maintaining the gC1q structure as well as having a pivotal role in IgG binding. Therefore, we examined the C1q-DC-SIGN interaction with Lys¹³⁶ substituted for Glu and Tyr¹⁷⁵ substituted for Lys. DC-SIGN and SIGN-R for ghB

with L136G resulted in nearly 60% of binding abolished (figure 4.15 and 4.17), suggesting that DC-SIGN binding to ghB overlaps with IgG. When DC-SIGN and SIGN-R were incubated with T175L, binding was reduced to 60-75% (figure 4.15 and 4.17) which was not surprising due to the role of Tyr¹⁷⁵ in stabilizing the gC1q structure.

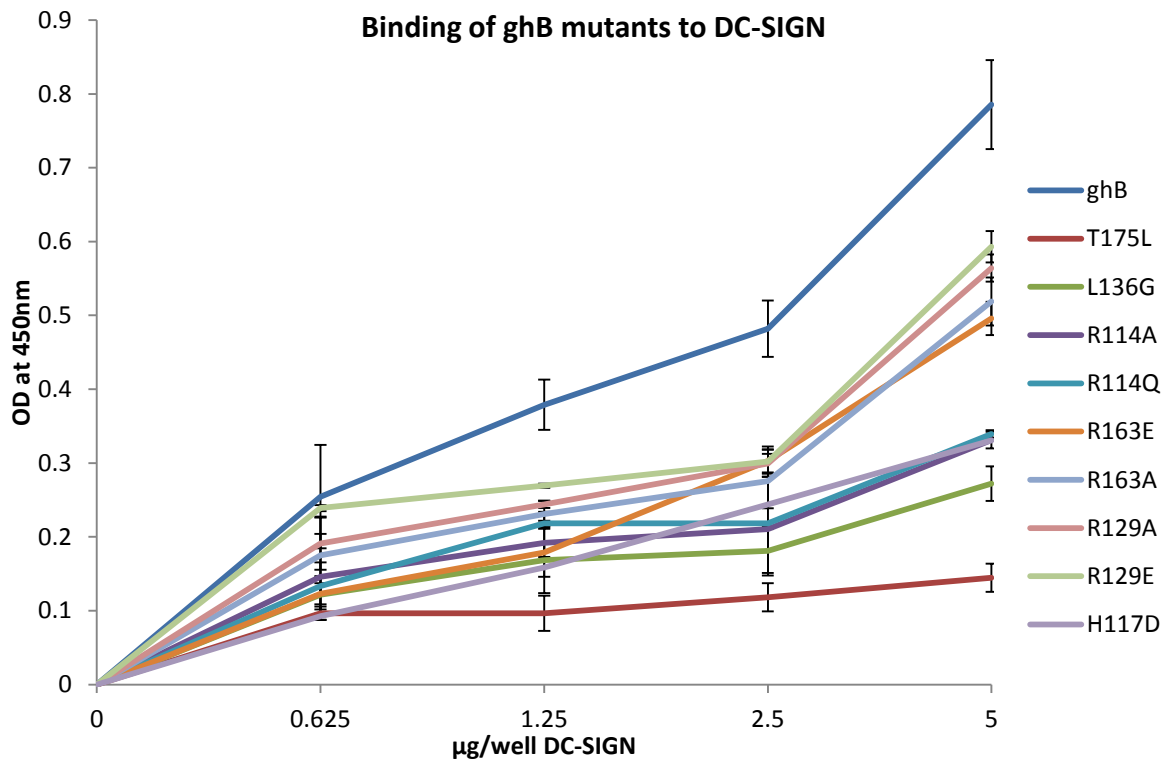


Figure 4.14: Binding of ghB mutants L136G, T175L, R114Q, R114A, R163A, R163E, R129E, R129A and H117D to DC-SIGN: Different concentrations of DC-SIGN Tetramer (5, 2.5, 1.25µg/well) was coated on microtitre wells in carbonate/bicarbonate buffer and incubated overnight at 4°C. Blocking was performed in 2% BSA for 2 h at 37°C and microtitre wells were washed. 2.5µg/well of ghB, T175L, L136G, R114A, R114Q, R163E, R163A, R129A, R129E and H117D were diluted in Calcium buffer and added in each well. Following incubation and washes, anti-MBP mAb and Protein-A HRP was used to detect bound protein. OPD was used to develop colour.

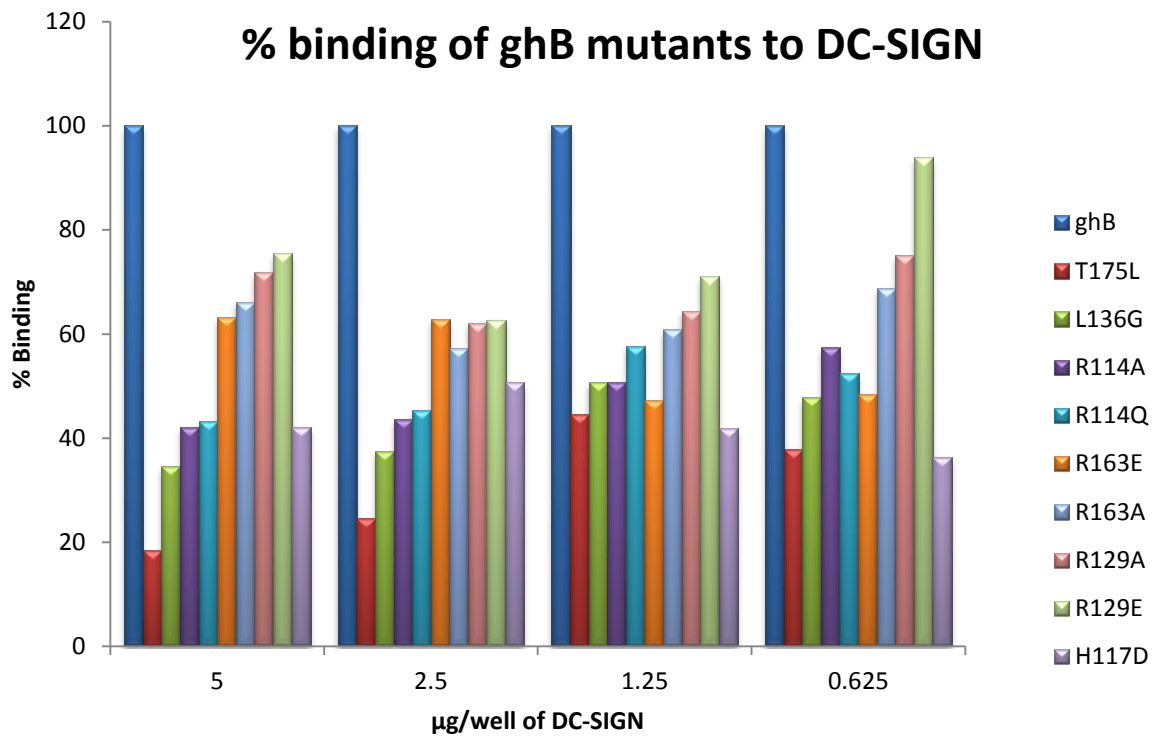


Figure 4.15: Percentage binding of ghB mutants to DC-SIGN: To qualitatively assess binding inhibitory properties of the ghB mutants, the percentage binding was worked out by using WT ghB as a 100% positive control at each concentration.

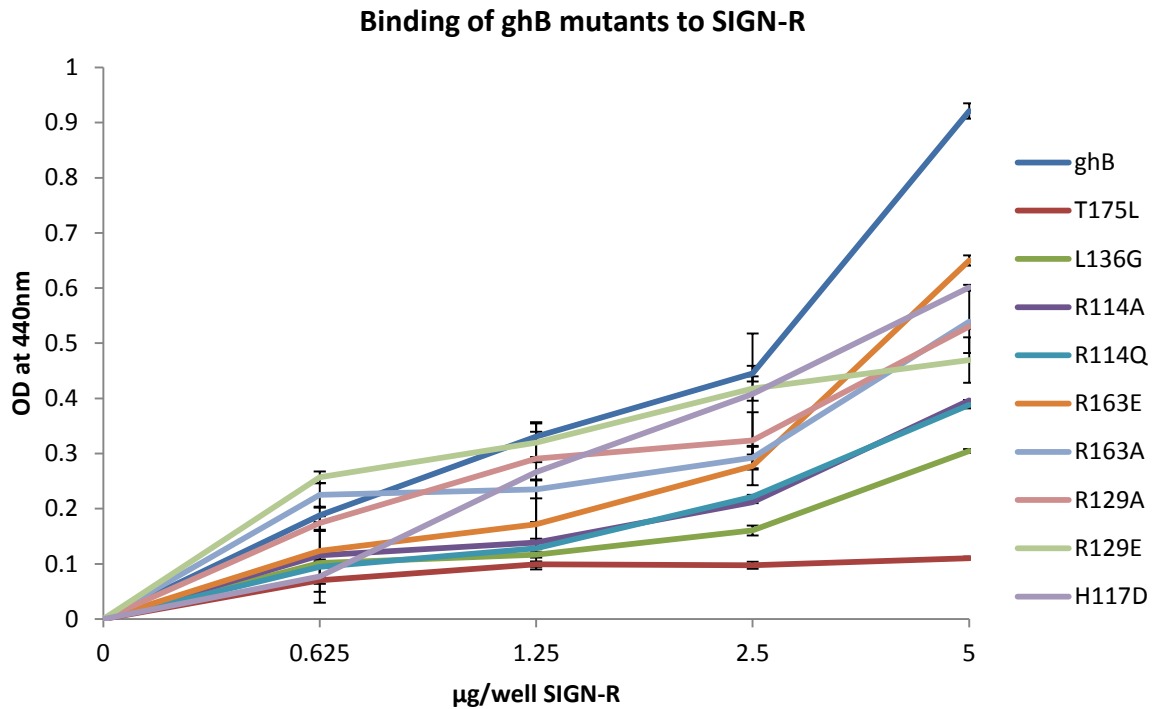


Figure 4.16: Binding of ghB mutants L136G, T175L, R114Q, R114A, R163A, R163E, R129E, R129A and H117D to SIGN-R: Different concentrations of DC-SIGN Tetramer (5, 2.5, 1.25µg/well) was coated on microtitre wells in carbonate buffer and incubated overnight at 4 degrees. Wells were blocked in 2% BSA in PBS for 2 hours at 37 degrees. Following this, wells were washed 3 times in PBS + 0.05% Tween and (2.5µg/well) of ghB, T175L, L136G, R114A, R114Q, R163E, R163A, R129A, R129E and H117D were added to each well in Calcium buffer and the plate was incubated at 37 degrees for 1.5 hours and at 4 degrees for 1.5 hours. Wells were washed again and bound protein was detected using mAb MBP following IgG-HRP (1:5000 dilution in PBS). Colour was developed using OPD and the plate was read at 415nm.

ELISA to examine binding of ghB substitution mutants to SIGN-R

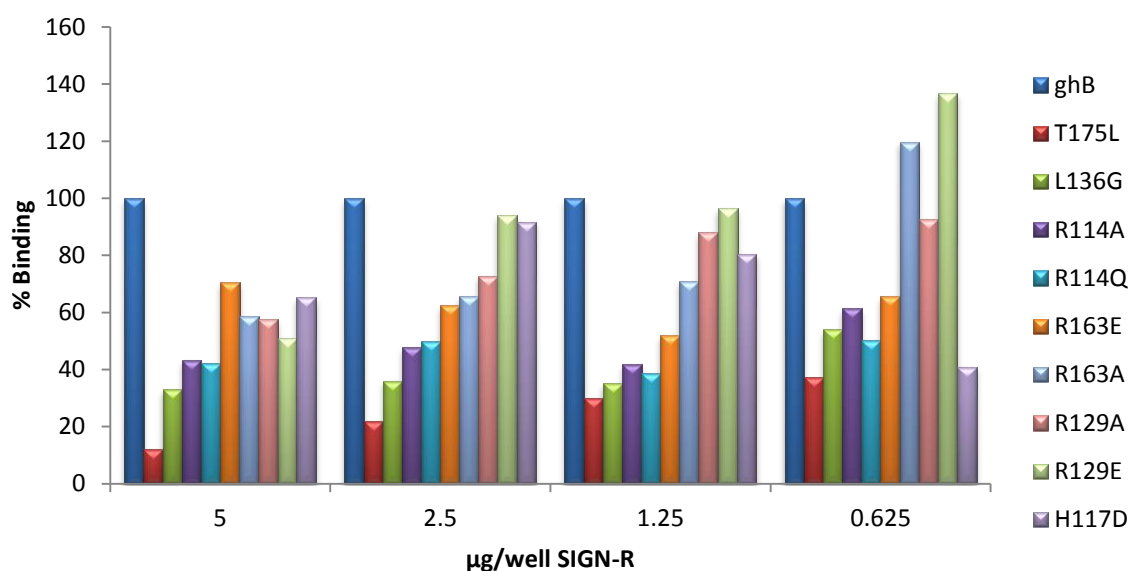


Figure 4.17: Percentage binding of ghB mutants to SIGN-R: To qualitatively assess binding inhibitory properties of the ghB mutants, the percentage binding was worked out by using WT ghB as a 100% positive control at each concentration

4.4.7 The contributions of ghC substitution mutants to DC-SIGN binding

ELISA to examine the interaction between ghC mutants and DC-SIGN showed that the substitution mutants H101A, R156E and L170E bound to DC-SIGN in a dose-dependent manner (figure 4.18). It appears that replacing Arg¹⁵⁶ with Glu of the ghC chain slightly strengthened binding to DC-SIGN with an increase in 15% (figure 4.19) suggesting that a Glu residue at this point is effective in attracting DC-SIGN. The ghC mutants H101A and L170E reduced binding to ghC by 15-20% (figure 4.19), suggesting that the contributions of His¹⁰¹ and Leu¹⁷⁰ are comparable in the DC-SIGN-C1q interaction.

ELISA to examine binding of ghC substitution mutants to DC-SIGN

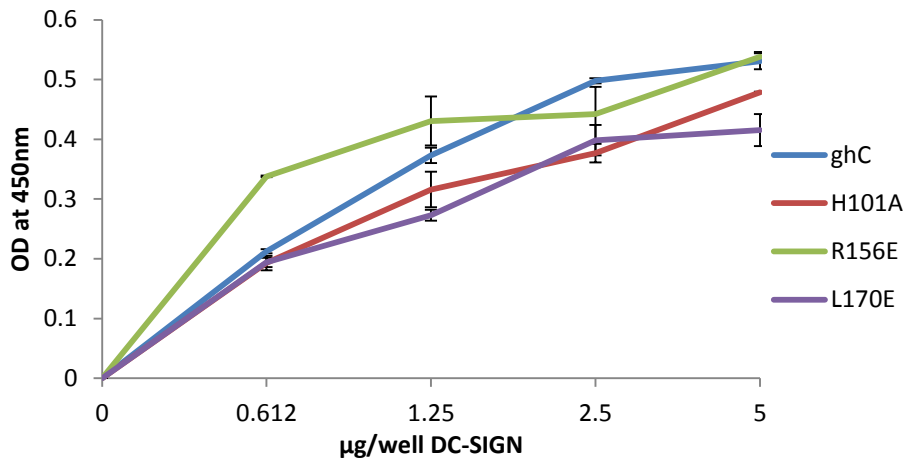


Figure 4.18: Binding of ghC mutants H101A, R156E and L170E to DC-SIGN: Different concentrations of DC-SIGN Tetramer (5, 2.5, 1.25µg/well) was coated on microtitre wells in carbonate buffer and incubated overnight at 4 degrees. Wells were blocked in 2% BSA in PBS for 2 hours at 37 degrees. Following this, wells were washed 3 times in PBS + 0.05% Tween and (2.5µg/well) of ghC, H101A, R156E and L170E were added to each well in Calcium buffer and the plate was incubated at 37 degrees for 1.5 hours and at 4 degrees for 1.5 hours. Wells were washed again and bound protein was detected using mAb MBP following IgG-HRP (1:5000 dilution in PBS). Colour was developed using OPD and the plate was read at 415nm **Percentage binding of ghC mutants to DC-SIGN:** To qualitatively assess binding inhibitory properties of the ghC mutants, the percentage binding was worked out by using WT ghC as a 100% positive control at each concentration.

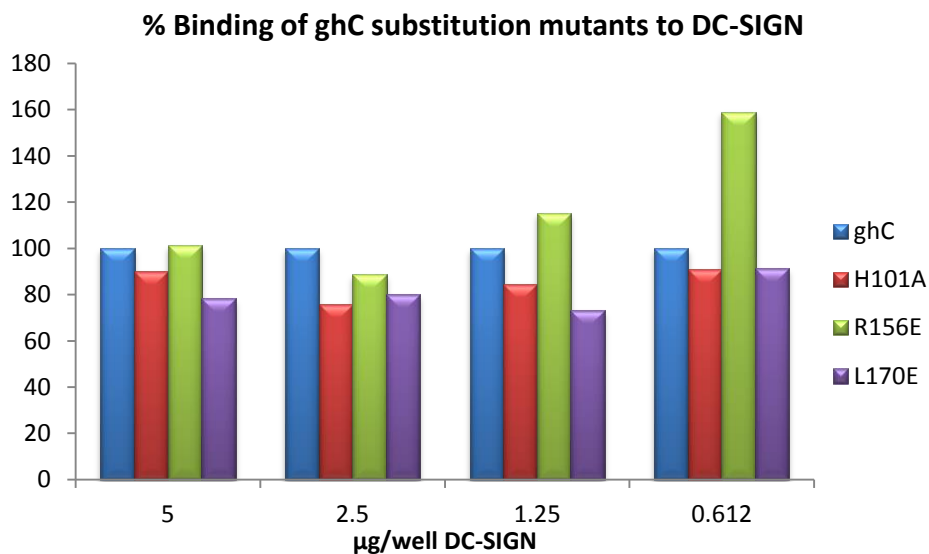


Figure 4.19: Percentage binding of ghC mutants to DC-SIGN: To qualitatively assess binding inhibitory properties of the ghC mutants, the percentage binding was worked out by using WT ghC as a 100% positive control at each concentration

4.4.8 The contributions of ghC mutants to SIGN-R

The ghC substitution mutants bound to SIGN-R in a dose dependent manner (figure 4.20). The substitution mutants H101A and R156E appeared to bind to SIGN-R as efficiently as its WT counterpart, whereas L170E showed a slight decrease in binding of 10% (figure 4.21).

ELISA to examine binding of ghC substitution mutants to SIGN-R

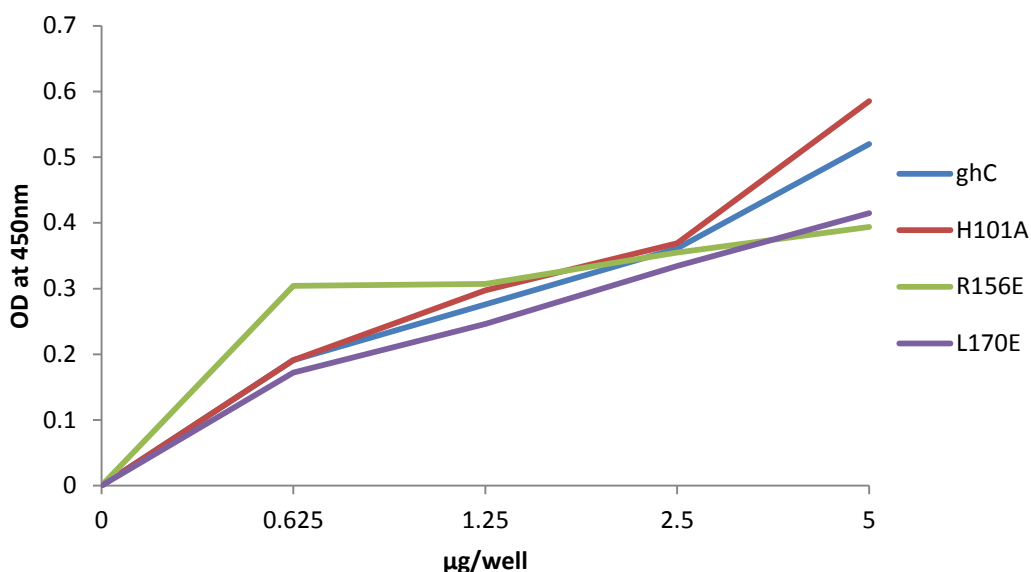


Figure 4.20: Binding of ghC mutants H101A, R156E and L170E to SIGN-R: Different concentrations of DC-SIGN Tetramer (5, 2.5, 1.25µg/well) was coated on microtitre wells in carbonate buffer and incubated overnight at 4 degrees. Wells were blocked in 2% BSA in PBS for 2 hours at 37 degrees. Following this, wells were washed 3 times in PBS + 0.05% Tween and (2.5µg/well) of ghC, H101A, R156E and L170E were added to each well in Calcium buffer and the plate was incubated at 37 degrees for 1.5 hours and at 4 degrees for 1.5 hours. Wells were washed again and bound protein was detected using mAb MBP following IgG-HRP (1:5000 dilution in PBS). Colour was developed using OPD and the plate was read at 415nm **Percentage binding of ghC mutants to SIGN-R:** To qualitatively assess binding inhibitory properties of the ghC mutants, the percentage binding was worked out by using WT ghC as a 100% positive control at each concentration

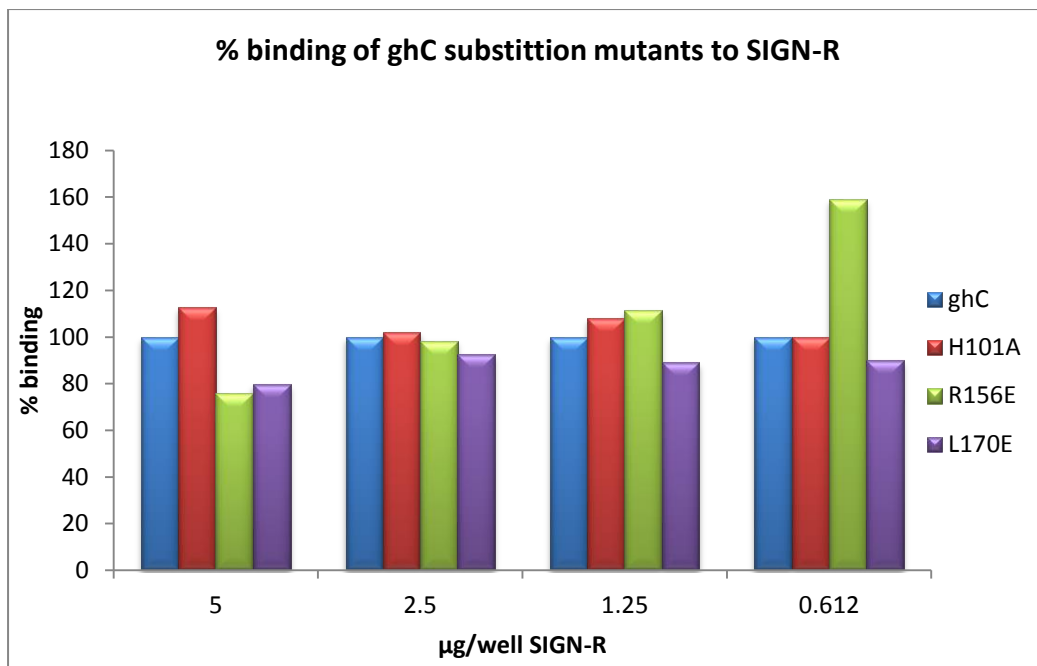


Figure 4.21: Percentage binding of ghC mutants to SIGN-R: To qualitatively assess binding inhibitory properties of the ghC mutants, the percentage binding was worked out by using WT ghC as a 100% positive control at each concentration

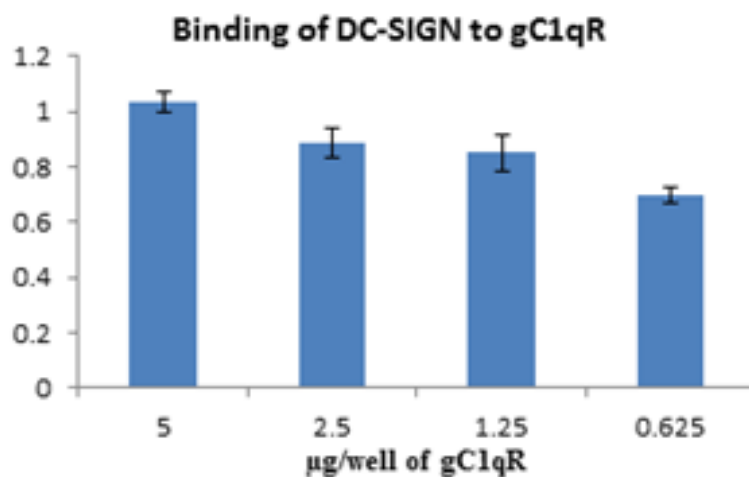
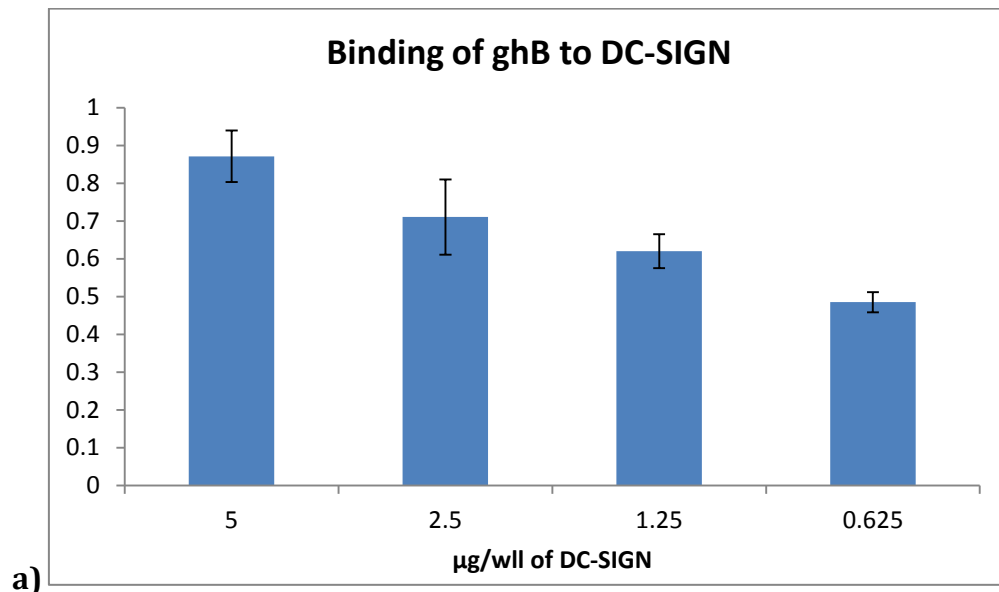


Figure 4.22) a) ELISA to show binding of ghB to DC-SIGN, b) ELISA to show binding of DC-SIGN to gC1qR. Different concentrations (5, 2.5, 1.25, 0.625µg/well) of either A) DC-SIGN or B) gC1qR were coated on microtitre wells in carbonate buffer and incubated overnight at +4°C. Contents were discarded and wells were washed in PBS + Tween 0.05%. 2.5µg of A) ghB or B) DC-SIGN in calcium buffer was added and the plate was incubated for 1.5 hours at 37°C and 1.5 hours at +4. Following repeated washing, plate A was detected using anti MBP followed by a IgG-HRP conjugate and pAb anti-DC-SIGN and Protein A HRP conjugate was used for plate B.

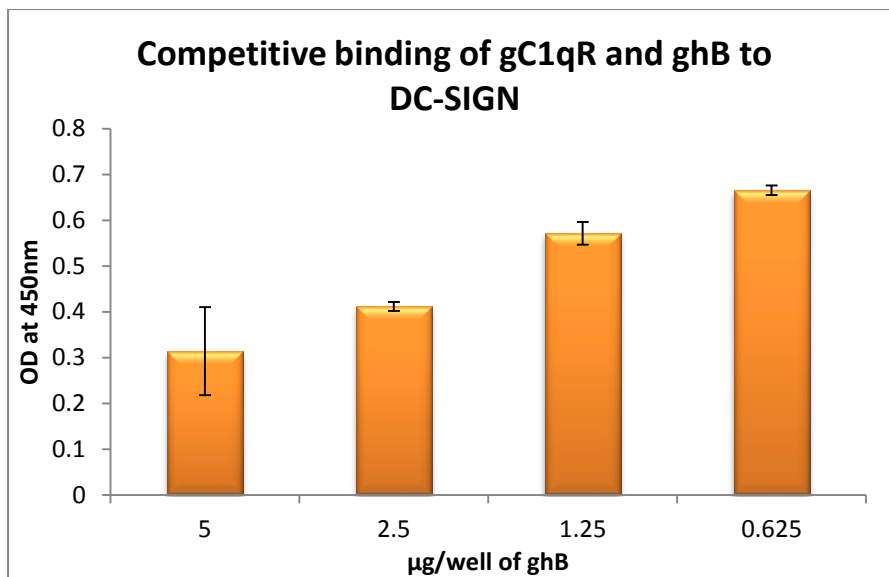


Figure 4.23: ELISA to assess whether gC1qR and ghB directly compete for the same binding site on DC-SIGN: (a) DC-SIGN was coated at 5µg/well overnight at +4. Following morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. Wells were then washed with PBS 0.05% tween 3 times and 5µg/well of gC1qR and different concentrations of ghB (5, 2.5, 1.25, 0.625µg/well) were added in buffer containing 5mM Calcium, 50mM Tris and 100mM NaCl. The plate was incubated at 37 degrees for 1.5 hours and +4 for 1.5 hours. Following repeated washes, bound gC1qR was probed using pAb rabbit anti-gc1qR and PA-HRP. Colour development was detected using OPD substrate. **(b)** Same concentrations of ghB were incubated with 5µg of gC1qR for 1 hour at 37 C and 30 minutes at +4 C prior to adding to wells.

4.4.9 gC1qR and ghB compete for the same binding site on DC-SIGN

Direct Binding ELISA determined that To further explore the possibility of gC1qR, C1q and DC-SIGN forming a trimeric complex, as shown on iDCs by Hosszu et al, we sought to see whether DC-SIGN holds a complimentary site for its two ligands. Since ghB is the preferential binder to DC-SIGN out of the three chains, we used this globular head protein to perform competitive assays. By incubating different concentrations of ghB and a steady concentration of gC1qR with DC-SIGN, probing with anti-gC1qR enabled us to see that as the concentration of ghB decreases, more gC1qR was able to bind to DC-SIGN (figure 4.23), thereby implying an overlapping site between the two ligands. Figure 4.22a shows that at a concentration of 5µg of DC-SIGN, 5µg is able to bind efficiently showing an OD of 0.8. However this binding appeared to drastically reduce when 5µg of gC1qR was added to compete with 5µg of ghB, as figure 4.23 shows the ability of ghB to interact with DC-SIGN was reduced by three folds.

4.5 Discussion

C1q is a charge pattern recognition protein that binds to a variety of ligands via its gC1q domain. Due to already being identified to interact with SIGN-R, Hosszu et al very recently has showed that C1q also recognizes its homologue DC-SIGN (Hosszu et al 2012). The availability of individual ghA, ghB and ghC modules has given us the opportunity to undertake structure-function studies and define whether individual globular head regions function as autonomous modules in this interaction with DC-SIGN and SIGN-R or whether a heterotrimeric structure of the gC1q domain is needed.

We have performed a series of experiments involving both the tetrameric forms of DC-SIGN and SIGN-R (comprising of the extracellular domain and CRD region) as well as the monomeric forms which only consists of the CRD region. We asked the question whether DC-SIGN and SIGN-R's binding site for C1q (and its globular head modules) lies within their CRD region or the α -helical neck region also plays a important role in these interactions. Both proteins have been shown to have an increased affinity for glycoproteins containing high mannose oligosaccharides, such as mannose (Appelmelk et al., 2003), gp120 (Geijtenbeek et al 2000) and ICAM-3 (Geijtenbeek et al., 2000a) with this CRD-mediated binding. Our results show that the individual globular head modules bind better to the tetrameric forms of DC-SIGN and SIGN-R as opposed to just the CRD region alone, indicating that the neck region is need to facilitate binding. It also suggests that you need a tatrameric structure for interaction. The neck region of DC-SIGN and SIGN-R interestingly differ from most α helical structures (Mitchell et al 2001) as the 23 amino acid repeats only show the first half of each repeat presenting a pattern of hydrophobic residues spaced at intervals, a feature that is abundant in most dimer and trimer coiled-coils (Mclachlan and Stewart, 1975). Therefore, this novel form of a tetrameric coiled-coil motif may prove important for structure-function studies.

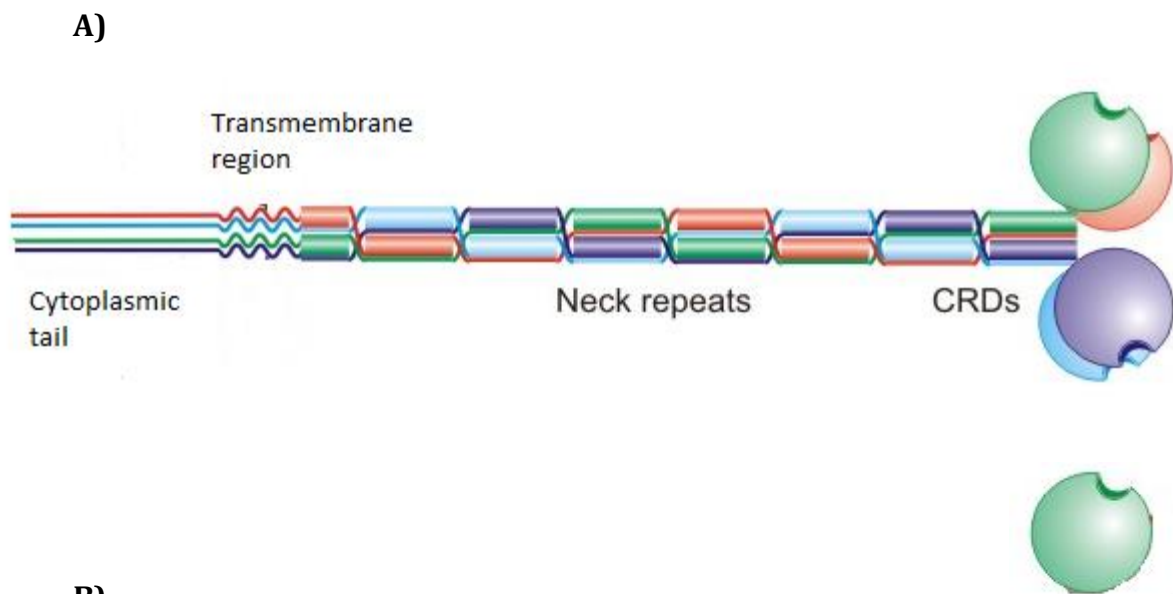


Figure 4.24: Schematic diagram to show structure of DC-SIGN: A) DC-SIGN Tetramer comprises of the extra cellular domain made up of the cytoplasmic tail, transmembrane region and neck. **B)** The DC-SIGN monomer made up of the CRD region alone (Mitchell et al 2001)

Our data also show that ghB out of the three globular domains binds preferentially to both DC-SIGN and SIGN-R. Previous studies have also identified ghB as a key module of the gC1qR domain in binding to ligands PTX3 and CRP (Roumenia et al 2006). Based on the 77% sequence similarity between these two homologues it is not surprising that the specificity within the globular domain is similar between the two. Physical studies also show that the specificity of these c-type lectins to identify glycoproteins lie largely within their CRD regions affinity to recognise specific oligosaccharides, as well as requiring calcium dependence (Stoiber et al., 1995), which is supported through their capacity of having similar ligand binding characteristics due to their almost identical structure (Soilleux et al., 2000). Another interesting feature made evident in our studies is that C1q as well as ghB is shown to bind with a greater affinity to SIGN-R than DC-SIGN. This is interesting as it reflects on the way the oligomers within the CRD region interact. The physical structure of SIGN-R employs a close spacing of two oligosaccharides which is remarkable as Mitchell et al has highlighted that this substantially leads to a higher increase in affinity for SIGN-R to binds ligands in comparison to DC-SIGN, which is clearly evident in this study. Our results clearly display that ghA, ghB and ghC all possess a greater binding affinity to SIGN-R tetramer in comparison to DC-SIGN Tetramer as well as SIGN-R monomer to DC-SIGN monomer. Moreover this confirms that the binding between this ligand (i.e C1q) and receptor is mediated via the CRD region.

The opportunity to express individual domains of ghA, ghB and ghC has allowed us to assess the modular nature of the gC1q domain. Furthermore, we were also able to express and purify globular head mutants (Gadjeva et al 2004) where single amino acid residues have been substituted which would help in identifying key residues that facilitate C1q's relationship with its ligands. These mutants were designed by analyzing the crystal structure due to their position within the domain and known residues to be important in binding. By performing ELISA with DC-SIGN and SIGN-R, we examined the interaction of ghB residue Arg¹⁶³, which was substituted with Glu and Ala, Arg¹¹⁴ substituted with Ala and Glutamine, Arg¹²⁹ with Ala and Glu, His¹¹⁷ with Asp, Leu¹³⁶ with Gly and Thr¹⁷⁵ with Leu. The role of Arginine residues have previously been shown to be important for the C1q-IgG interaction (Burton et al 1980), which is of great interest as Hosszu et al has suggested that DC-SIGN binds to C1q via its IgG binding site. In addition

to this, recent epitope mapping analysis has identified additional Arg residues of the ghB chain, Arg¹⁰⁸ and Arg¹⁰⁹ to be involved in the initial stage of recognizing IgG, as well as contributing to its final binding (Gadjeva et al 2008). Our results also support the significance of Arg residues, clearly showing that Arg¹¹⁴, which is implicated as a vital residue in IgG binding is also important in DC-SIGN and SIGN-R binding, with figure 4.17 shows a 60% reduction in binding when compared to WT ghB at the highest concentration of 2.5µg/well. Our data shows that substitutions of Arg¹²⁹ and Arg¹⁶³ with either Glu or Ala did not make a significant difference in binding to DC-SIGN when comparing both mutants capacity to interact. Both mutants showed a decrease in 30% (figure 4.17). The same was observed when substituting Arg¹¹⁴ with the polar residue Glutamine and hydrophobic residue Ala, binding between the two mutants was both abolished at the same maximum ability of 80%. This, in turn, highlights a very important role for Arginine¹¹⁴ of human C1q ghB chain in maintaining the C1q-DC-SIGN interaction. This is consistent with Kojouharova et al 2004 who showed that residue Arg¹¹⁴ is the most important residue in C1q-IgG binding.

Our mutational studies have indicated, that residue Tyr¹⁷⁵ is also critical for C1q-DC-SIGN/SIGN-R binding. Figure 4.17 displays a dramatic reduction (82% for DC-SIGN and 90% for SIGN-R) following substituting of Tyr for Leu. This is not the first time Tyr¹⁷⁵ has been shown to be a critical residue in gC1q binding, Gadjeva et al (2006) have shown that this residue mainly constitutes C1q binding to IgM. Over all our binding studies suggest that Tyr¹⁷⁵ of ghB is critical for the C1q-DC-SIGN and C1q-SIGN-R interaction with Arg¹¹⁴ making important contributions.

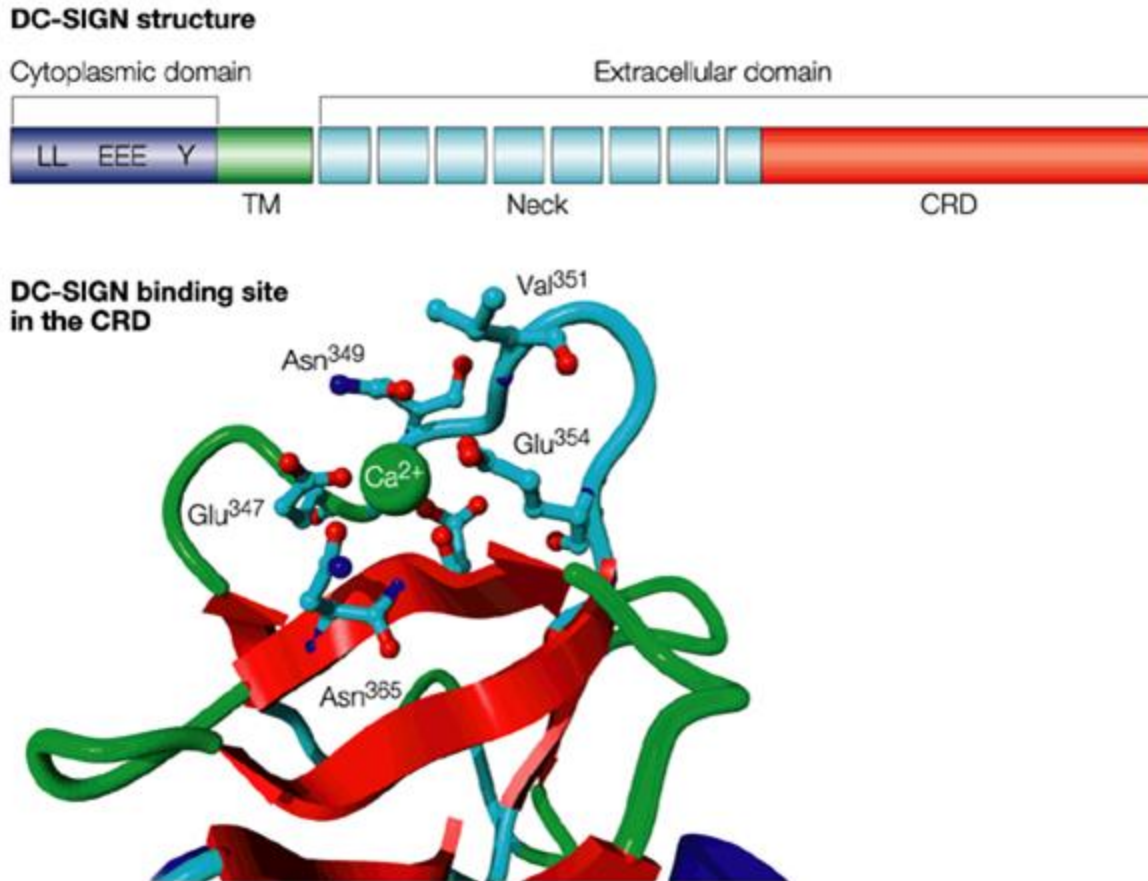


Figure 4.25: Structural representation of DC-SIGN: DC-SIGN is a C-type lectin consisting of a CRD involved in binding carbohydrate residues in highly glycosylated ligands. The carbohydrate ligands are able to interact with Ca²⁺ through hydroxyl groups with amino acids Glu and Asn assisting this binding. The CRD region is made up of 12 β strands, 2 α helices and 3 disulphide bridges. A loop protruding from the protein surface forms two Ca⁺ sites. One of the Ca⁺ sites is essential for the conformation of the CRD whereas the other domain contains Glu³⁴⁷, Asn³⁴⁹, Glu³⁵⁴ and Asn³⁶⁵ which are involved in recognizing specific carbohydrate structures. Mutation of these sites leads to a loss in ligand binding (Geutenbeek et al, 2002). The CRD of DC-SIGN is separated from the transmembrane region by a neck comprised of 7 repeats. The neck is required for oligomerization.

Chapter 5:

Identification of the gC1qR sites for the HIV-1 viral envelope protein gp41 and the HCV core protein: Implications in viral-specific pathogenesis and therapy

5.1 Abstract

gC1qR is increasingly being recognized as a major pathogen-associated molecular pattern recognition receptor (PRR). A wide range of bacterial and viral ligands, which bind gC1qR, are able to use it to either suppress the host's immune response for their survival, or to gain access into cells to initiate viral-specific disease. Of the extensive array of viral ligands that have affinity for gC1qR, the HIV-1 envelope glycoprotein gp41 and the core protein of HCV, are of major interest as they are known to contribute to the high morbidity and mortality caused by these pathogens. While the HCV core protein uses gC1qR on T cells to suppress their proliferation resulting in a significantly diminished immune response, gp41 employs gC1qR to induce surface expression of the NK cell ligand, NKp44L, on uninfected CD4⁺ T cells, thus rendering them susceptible to autologous destruction by NKp44R expressing NK cells. Because of the potential for the design of peptide-based or antibody-based therapeutic options, this chapter examines gC1qR interaction sites for these pathogen-associated molecular ligands. The binding of each viral ligand to wild type gC1qR and 10 gC1qR deletion mutants was examined in a ELISA. The results identify two major HCV core protein sites on a domain of gC1qR comprising of residues 144-148 and 196-202. Domain 196-202 in turn, is located in the last half of the larger gC1qR segment encoded by exons IV-VI (residues 159-282), which was proposed previously to contain the site for HCV core protein. The gC1qR site for gp41 on the other hand, was found to be in a highly conserved region encoded by exon IV and comprises of residues 174-180. Interestingly, gC1qR residues 174-180 also contain the cell surface-binding site for soluble gC1qR (sgC1qR), which can bind to the cell surface in an autocrine/paracrine manner. The identification of the precise sites for these important pathogen associated ligands could provide additional targets for the design of peptide-based or antigen-based therapeutic strategies.

5.2 Introduction

The receptor for the globular heads of C1q, gC1qR/p33, (also referred to as a C1q binding protein; gC1qBP) is a ubiquitously distributed highly acidic (pI 4.15), multifunctional cellular protein, which modulates a number of immunological functions including infection, inflammation, autoimmunity, and cancer. In addition to its known plasma ligands (C1q, High Molecular weight Kininogen (HK), fibrinogen, thrombin and vitronectin), gC1qR is able to bind a diverse range of bacterial (Nguyen et al., 2000) (Ghebrehiwet et al., 2007) and viral-associated molecular ligands (Wang et al., 1997, Yu et al., 1995) (Luo et al., 1994, Szabo et al., 2001, Fausther-Bovendo et al., 2010, Bruni and Roizman, 1996, Matthews and Russell, 1998, Kittlesen et al., 2000, Mohan et al., 2002, Choi et al., 2009, Watthanasurorot et al., 2010). Thus gC1qR is being considered an important PRR. The core protein of the HCV and the envelope glycoprotein of the HIV-1 gp41 are two examples of viral associated molecular ligands that employ gC1qR to induce immunosuppression by targeting CD4⁺ T cells. The HCV core protein binds gC1qR on T cells and inhibits their proliferation (Kittlesen et al., 2000) in a manner that is similar to the C1q mediated anti-proliferative response (Chen et al., 1994). Suppression of the host immune response contributes to a high rate of viral persistence and disease progression (Kittlesen et al., 2000, Yao et al., 2004). Similarly, the HIV-1 glycoprotein gp41, not only binds to the globular head domain of C1q via the 3S motif of gp41 (GIFGCSGKLICTT), but this interaction leads to activation of the classical pathway (Thielens et al, 1993; Quinkal et al, 1999). gp41 is also known to modulate T cell destruction thereby contributing to the persistently low level of T cells seen during the late phase of HIV-1 infection (Fausther-Bovendo et al., 2010; Caffrey, 2001). The 3S motif of HIV-1 gp41 corresponding to residues 601-620 (PWNASWSNKSLDDIW) has been shown to bind to gC1qR on uninfected bystander CD4⁺ T cells and induce surface expression of NKp44L, a cellular ligand for the NK receptor, NKp44, rendering them susceptible to autologous NK killing (Fausther-Bovendo et al., 2010). Because molecules or peptides that inhibit the interaction between PAMPs and gC1qR can potentially be used as targets for therapeutic interventions, the present studies were undertaken to map out and refine the precise gC1qR interaction sites for these important molecules.

5.2.1 Interaction of Gp41 with complement

Gp41 is also shown to bind to C1q. The loop region of gp41 at residues 601-613 is involved in this interaction (Thielens et al., 1993; Ebenbichler et al., 1991). It is known that retrovirus isolates from animals are able to undergo lysis by complement proteins in an antibody-independent manner (Bartholomew et al., 1978). However HIV-1 does not behave in the same manner when bound to C1q. Instead of undergoing complement mediated lysis of the infected cell, this interaction results in the deposition of C3b on the infected cell surface, which aids in the recruitment of immune cells such as phagocytes. However, it is not known yet whether this mechanism lessens the intensity of infection HIV (Solder et al., 1989). The behaviour of complement receptor bearing cells with HIV-1 has been studied by Reisinger et al (1990). Experiments performed on the monocytic cell line U937 showed that HIV-1 in fact uses complement receptors as a port of entry. Low doses of HIV-1 enhanced infection of these U937 cells in the presence of complement. This observation has been further supported by the fact that addition of the mAbs (anti-CD4) and (anti-CR3) reduced HIV infection (Reisinger et al, 1990)

The inability of complement to lyse the virus could be due to certain factors abundant in an individual that work to separate 'self' from 'non-self'. Such factors include decay accelerating factor (DAF; CD55), which serves to protect the body's host tissue against the complement by binding C3b, thereby disassembling the C3 convertase complex and inhibiting activation of the membrane attack complex (MAC), (Caras et al., 1987). Such factors could be embedded in the HIV membrane which would effectively provide a protective mechanism against complement mediated lysis.

The HIV loop region possesses many properties, which support the above mentioned interaction with C1q. Firstly, the loop region displays a lack of charged residues which is evident in literature that charged residues are less involved in protein-protein interactions (Zhao et al., 2011). It has been proposed that C1q recognises gp41 through hydrophobic interactions (Quinkal et al., 1999). The loop region is rich in hydrophobic clusters (Caffrey, 2001) (Only 4 hydrophilic residues are present out of 30 residue sequence) (Caffrey, 2001). Another striking feature observed in the loop region is the formation of a cleft, which is the likely docking site for the gC1q domain (Caffrey 2001). It has been shown that the ghA module from the gC1q region is specific to the loop

region of HIV-1 gp41 protein (Kishore et al., 2003). Quinkal et al 1999 used synthetic peptides corresponding to residues 601-613 of the gp41 loop and a mutational form by substituting a single amino acid in each sequence. As the wild-type peptide 601-613 was able inhibit binding of gp41 to C1q, the mutant peptides Ile602, Trp603, Lys608 and Ile610 failed to inhibit the binding of gp41 to C1q, thereby implying that these residues are key in the interaction between gp41 and C1q, (Quinkal et al,1999).

5.2.2 Role of gp41 & gC1qR in HIV infection

Recently, it has been established that gp41 brings about its insertion of viral genome into its target cell via binding to gC1qR, which is expressed on the surface of CD4⁺ T cells (Fausther-Bovendo et al., 2010). The 3S motif of gp41 consisting of 6 sequences (SWSNKS) located on residues 618-623 is central to this interaction (Vieillard et al., 2005). Initially the CD4⁺ T cell loss was thought to arise from cytotoxic killing of the cells infected with the HIV-1 virus. However, research has evidently exploited that death of these T cells is not in fact due to their infection by cytotoxic killing (Finkel et al., 1995) but bought upon by the activation of signaling pathways to initiate apoptosis in uninfected cells (Alimonti et al., 2003).

It has emerged that such a signaling cascade results with the cell surface translocation of the Natural Killer (NK) ligand NKP44L , which binds to its counterpart cytotoxic receptor NKP44 causing T cells to become susceptible to NK lysis (Ward et al., 2007). NK lysis involves the cells releasing small cytoplasmic granules of proteins that initiate the target cell for death by apoptosis.

Binding of the 3S motif with gC1qR activates class I phosphatidylinositol 3-kinase PI3Ks, a group of enzymes involved in regulation of cell growth (Engelman et al., 2006). These indirectly activate the Rho GTPase family member Rac. This, in turn, triggers the membrane bound enzyme complex NADPH Oxidase, whose activation is dependent upon the action of a Rac protein (Heyworth et al., 1993). NADPH oxidase stimulates T cells to release H₂O₂ which induces GTP hydrolysis of TC10, another member of the Rho family of GTPases, into its active form. TC10 is then involved in bearing the exocytosis of NKP44L within its vesicles to the cell surface. It is by this mechanism that gp41 is able

to initiate the translocation of NKP44L, which predisposes healthy CD4 T cells to NK mediated lysis, leading to the depletion of T-cells observed in HIV infection.

It has also been proposed that the involvement of the 3S Motif with NKP44L lies solely within the expression of this ligand on the cell surface, not its synthesis. CD4-T cells unstimulated by the 3S motif were shown to retain NKP44L in their cytoplasm with the inability to translocate it which illustrates that the 3S motif is only able to stimulate the translocation of pre-existing NKP44L from the cytoplasm to the cell surface (Fausther-Bovendo et al., 2010)

Fausther et al have confirmed that gC1qR is the receptor for the 3S Motif. In addition, CD4⁺ T cells pre-treated with anti gC1qR mAbs were sufficient to inhibit the binding of the 3S-motif, which in turn inhibited levels of NKP44L in T cells (Fausther-Bovendo et al., 2010).

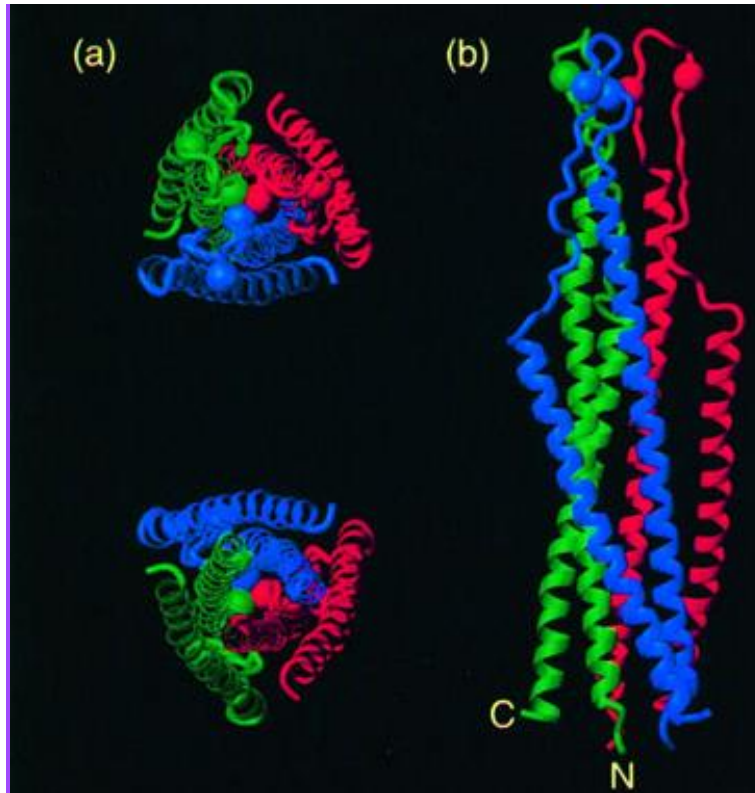


Figure 5.1: Structure of gp41: HIV-1 gp41 is composed of a trimeric configuration of two peptides, N36 and C34 derived from the N and C terminal regions of the ectodomain of gp41. These peptides come together to form a 6 helical bundle. The N36 helix are packed interiorly, aligned in a coiled manner parallel to each other, whereas the C-terminal helix is packed anti-parallel to the N36 peptides, on the surface of this trimer (Chan et al., 1997) **A)** Axial view looking down the 3-fold axis of the trimer, the N terminal helix is packed inside surrounded by C-terminal helix. **B)** Lateral view of trimer (Chen et al, 1997)

5.2.3 Gp41 structure and relevance in host-pathogen interaction

Gp41 and gp120 are held together by non-covalent interactions, the importance of this weak contact between the two molecules provides gp120 with its flexibility in order to undergo its change in shape, as well as arrest gp41 transitions after binding to its target cell (figure 5.4) (Pancera et al., 2009). The structural elements that make up gp120 include the β sandwich, the outer domain, and the V1/V2 loops (figure 5.2). The gp120 termini and 7 stranded β sandwich is what appears to stabilise the gp120 – gp41 interaction.

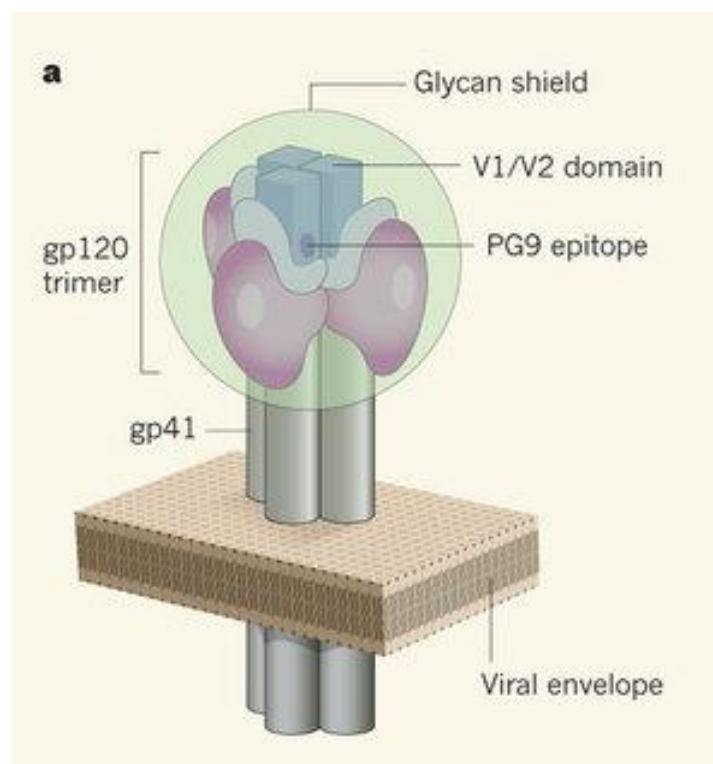


Figure 5.2: gp41-gp120 interaction: Three monomers of gp120 make up the trimeric structure represented in purple and bind to 3 molecules of gp41 to form a trimeric complex (Burton et al, 2004)

Structurally, the gp41 subunit is composed of 3 domains consisting of an ectodomain, a cytoplasmic domain, and a membrane spanning domain (figure 5.3) (Long et al., 2011). The ectodomain is composed of a N-terminal and C-terminal region (figure 5.1). The N-terminal contains the fusion peptide which inserts into the target membrane at the start of the membrane fusion process, (Stegmann et al., 1991) a polar region and a MPER

(Membrane Proximal External Region). The MPER is a highly conserved sequence made up of 24 amino acids and participates in promoting fusogenicity and virus infectivity (Munoz-Barroso et al., 1999).

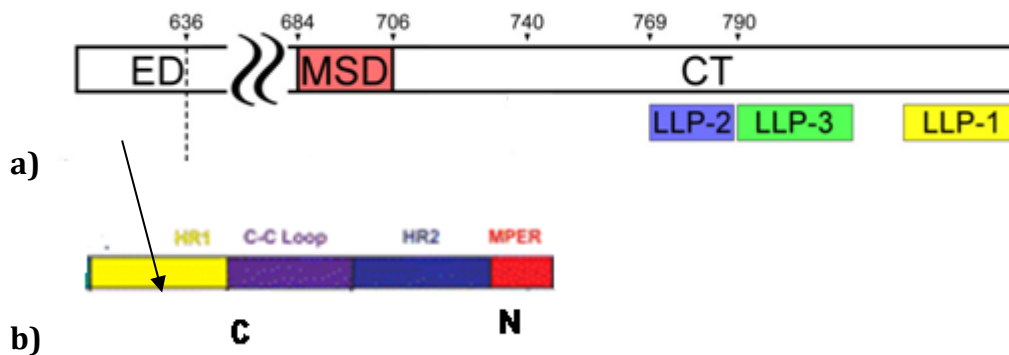


Figure 5.3: Model of gp41 domains: a) three domains are present in gp41, the ectodomain, the MSD (Membrane Spanning Domain) and the Cytoplasmic domain within which are the lentiviral lytic peptides (LLP). Left of the vertical dashed line indicates the N-terminal whereas to the left of it represents the C terminal. b) Composition of ectodomain shows N-terminal region containing MPER and C-terminal consisting of Heptad repeats regions 1 and 2 held together by the loop (Frey et al., 2010)

The C-terminal is composed of 2 coiled heptad repeat regions called HR1 and HR2 (figure 5.3) (Munoz-Barroso et al., 1999) that form an α -helical structure that is hydrophobic in nature and held together by a disulphide bridge within a hydrophilic loop (Melikyan, 2008). Three HR1 helices are present which are aligned in parallel to one another and fold over a hydrophobic groove. Positioned anti-parallel to this are 3 motifs of HR2, binding of the fusion peptide to the host cell causes HR2 to bind back, creating a hair-pin structure, which together gives rise to a six helical bundle that creates close enough contact between the viral membrane and cell membrane. It is this interaction that allows fusion to occur. The cytoplasmic domain contains 3 amphipathic α -helical sequences, known as lentivirus lytic peptide 2 (LLP2), LLP-3 and LLP-1, all of which are membrane interacting (Yang et al., 2010). Both LLP1 and LLP2 contain Arg residues on one face of their α -helix which makes them positively charged. LLP segments have been associated with envelope fusogenicity (Kalia et al., 2003), stability of the envelope protein (Lee et al., 2002) and multimerisation, where it was shown that LLP1 and LLP2 play a vital role in assembling the cytoplasmic domain into a multimeric

structure, which is in confluence with previous findings stating that the multimeric form of the cytoplasmic domain is key in virus infectivity (Lee et al., 2000). It has also been demonstrated that the LLP segments bind membranes (Chen et al., 2001) and induce cytolytic activity (Miller et al., 1993). Substitution of Arg residues in LLP-1 with Glu disrupts cell lysis activity. It has recently been proposed that LLP2 becomes exposed on the cell surface during fusion of the virus with the host cell (Lu et al., 2008). The cytoplasmic region also contains two cysteine residues that have been implicated in targeting the Envelope to the lipid rafts (Rouso et al., 2000) and in association with this, LLP-1 has also been suggested to play a part in this anchorage, as a mutated form of LLP-1 reduced attachment of the envelope to the lipid rafts.

Lastly the MSD is composed of a membrane spanning α - helix made up of 25 amino acid residues (Haffar et al., 1988) that anchor the envelope into the lipid bilayer. The highly conserved arrangement of the MSD implies that it plays a role in the envelope function and this statement has been supported by experiments showing that the insertion of mutations in the core region of this domain affected Envelope mediated fusion (Shang et al., 2008).

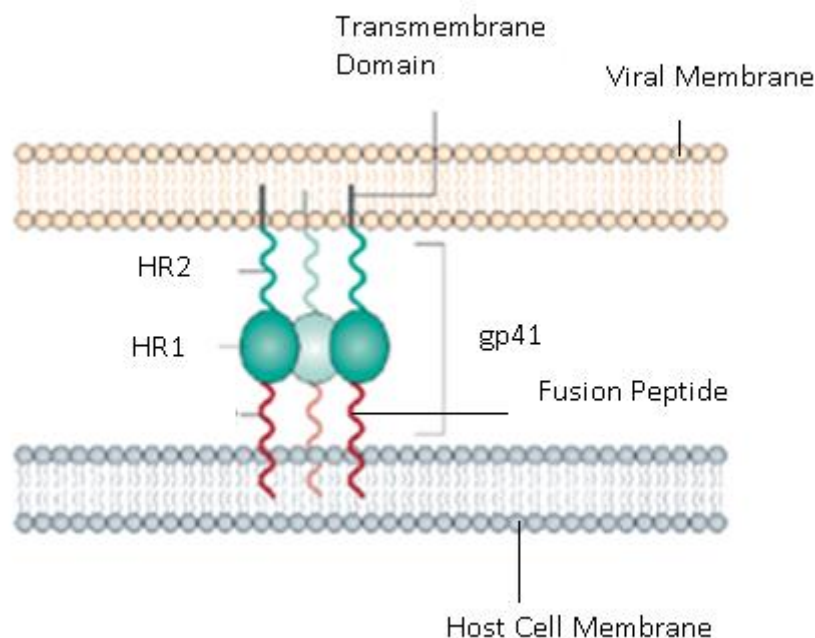


Figure 5.4: Structure of gp41 interacting with host cell membrane: As the fusion peptide binds to the host cell membrane, the three motifs of HR2 are able to bind back bringing the viral membrane in close proximity to the host cell membrane to assist fusion of viral content (Miller et al., 2005)

5.3 Materials and Methods

5.3.1 Chemicals and general reagents

The following reagents and chemicals were purchased or obtained from the sources indicated: Dulbecco's PBS (D-PBS) with and without calcium and magnesium (Mediatech Inc, Manassas, VA); Dulbecco's Modified Eagles Medium (DMEM); RPMI 1640, 100x Penicillin/Streptomycin, (GIBCO-Invitrogen, Grand Island, NY); heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT); human serum albumin (HSA) (Immuno-US, Rochester, MI); *p*-nitrophenyl phosphate (pNPP) (Pierce, Rockford, IL); and Immu-Mount (Thermo Fisher, Waltham, MA). Alexa 488- or Alexa 594-Streptavidin, Alexa 488- or Alexa 594-F(ab')₂, goat anti mouse or anti rabbit; FITC-conjugated goat anti-mouse IgG F(ab')₂ or sheep anti-rabbit IgG F(ab')₂ (Invitrogen, Carlsbad, CA); and alkaline phosphatase (AP)-conjugated rabbit anti-goat IgG (Pierce), and C1q (CompTech), D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK)—a specific thrombin inhibitor (Sigma-Adlrich).

5.3.2 Expression of various versions of recombinant gC1qR proteins

The strategy for the construction of plasmids containing the glutathione-S-transferase (GST)-gC1qR wild type (WT), as well as several important deletion (Δ) mutants lacking highly charged domains selected on the basis of their prominent position in the crystal structure (Jiang et al., 1999) have been described. (Ghebrehiwet et al., 2002; Ghebrehiwet et al., 2001). In addition, a point mutation that projects from the S face in the crystal structure has also been generated with the idea that its conspicuous projection might signify its biological relevance (Ghebrehiwet et al., 2001). The fusion products were purified on glutathione-Sepharose 4B column, then cleaved by thrombin (3.2 μ g /ml), and the GST-free gC1qR proteins purified on fast protein liquid chromatography (FPLC, Pharmacia) using a Mono-Q ion exchange column. After verification by ELISA and Western blotting, the single peak containing the gC1qR protein was pooled, concentrated to 1-2 mg/ml and stored at -80° C in the presence of 50 nM PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone)—a specific thrombin inhibitor (Sigma Adlrich). To estimate the organization and integrity of each recombinant protein, gel filtration of purified gC1qR (wild Type, WT) as well as the various deletion mutants was carried out by analytical gel filtration Superose column of

(Ghebrehiwet et al., 2001). With the exception of those lacking residues 74-95, 204-218, and 212-223, all of the deletions mutants displayed a properly folded trimer (Figure 5.5).

5.3.3 Expression and purification of gC1qR deletion mutants

The plasmids generated for expressing the WT gC1qR used in these studies as well as the gC1qR deletion mutants were constructed as GST-fusion product in the vector pGex-2T by Ghebrehiwet et al 1994. The GST-gC1qR fusion products were cleaved by thrombin (3.2µg/ml) and the gC1qR was purified using a Mono-Q ion exchange column. The single peak was pooled and frozen at -20

5.3.4 Purification of recombinant WT gC1qR and gC1qR deletion mutants

The cDNA of WT gC1qR and the deletion mutants were subcloned to the glutathione S-transferase gene in the plasmid pGex-2T to be expressed as fusion products with the GST part at the N terminus and the gC1qR at the C terminus. The plasmid was transformed in *e.coli* and purified on a glutathione-Sepharose column. The purified gC1qR fusion proteins were then cleaved by thrombin and the released gC1qR proteins were purified on a Mono-Q ion-exchange column

5.3.5 Single point mutagenesis

The solution face of gC1qR in the crystal structure displays a domain made up of a single residue Trp 233 that project conspicuously. A single residue mutation was carried out on Trp 233 where the codon TGG was mutated by a single base change to GGG (Gly) by double strand mutagenesis and transformed into *E.coli* and purified as the other deletion mutants.

5.3.6 Trimer Formation

The organization and ability of each gC1qR deletion mutant as well as WT to form a trimer was analysed using gel filtration. A Superose beads column (1.4cm x 30cm) was equilibrated in a buffer made up of 50mM Tris-HCL buffer pH 7.4 and 1mM EDTA (Ghebrehiwet et al 1994). The flow rate was adjusted to 0.2ml/min and the column was calibrated with two proteins Carbonic Anhydrase (29kDa) and BSA (65Kda) due to them having similar molecular weight to the monomeric and trimeric forms of gC1qR. The

peak elution of these two proteins was noted and used as the control to compare, against the elution time of each gC1qR recombinant protein.

5.3.7 SDS-PAGE and Western blot analysis

SDS-PAGE was performed on a 1.5 mm thick slab gels according to the method of Laemmli (Laemmli, 1970) with samples being run unreduced or reduced and alkylated by boiling for 5 min in the presence of 0.1 M dithiothreitol and 0.2 M iodoacetamide. After electrophoresis, the gels were stained either with Coomassie Brilliant Blue or silver stain, destained and dried. Samples for Western blot analysis were first run on SDS-PAGE as, and above then electrotransferred to polyvinyl difluoride (PVDF) nitrocellulose membranes, blocked with 5% non-fat milk containing TBST (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20). The bound proteins were probed with an appropriate dilution of target-specific antibodies. The bound antibodies in turn were visualized by chemiluminescence horseradish peroxidase-conjugated species-specific antibody followed by reaction with 4-chloro-1-naphthol substrate.

5.3.8 Proteins and antibodies

Purified recombinant gp41, CD4 as well as monoclonal antibodies to gp41 and CD4 were obtained from the NIH AIDS repository Program (Germantown, MD). HCV core protein was purchased from Abcam (Cambridge, MA) and rabbit anti-fibrinogen antibodies were a generous gift from Dr. Dennis Galanakis (Stony Brook University, NY). Antibodies to recombinant human gC1qR represent part of the anti-gC1qR antibody databank in the laboratory (Cummings et al., 2009). Affinity purified rabbit anti-gC1qR peptide 174-180, and a synthetic peptide corresponding to the 3S motif of gp41 were purchased (GenScript, Piscataway, NJ). Monoclonal antibodies to *E. coli* MBP (maltose binding protein) were purchased from Sigma. MBP -fused- recombinant globular head regions of human C1q: ghA, ghB, and ghC, were expressed and purified as experiments described previously (Kishore et al 2003). The ghA substitution mutants of globular head modules were generated as described earlier (Kojouharova et al 2004).

5.3.9 ELISA to examine binding of HCV core protein and HIV-1 gp41 to gC1qR deletion mutants

The ability of the various gC1qR proteins to bind to HCV core protein or HIV-1 gp41 was assessed by ELISA. Microtiter wells were coated in duplicate (90 min, room temp or overnight, 4°C) with 100 µl of either 1µg/ml HCV core protein, gp41, or BSA, in carbonate/bi-carbonate buffer, pH 9.6 (15 mM Na₂CO₃ and 35 mM NaHCO₃). The unbound protein was removed; the wells washed twice with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween -20), and the unreacted sites blocked by incubation (30 min, room temp) with 300 µl of 5% non-fat dry milk or blotto (10 mg/ml casein in TBS, pH 8.0). After washing (2 x with TBST), the microtiter plate bound HCV or gp41 was incubated (60 min, room temp.) with various concentrations of biotinylated wild type gC1qR ranging from 0-5 µg/ml. This was followed by sequential reaction (60 min each, room temp) with alkaline-phosphatase conjugated streptavidin and pNPP. After firmly establishing dose-dependent binding (n=3), experiments were repeated as above to compare the binding of either 5 µg/ml biotinylated WT gC1qR or deletion (Δ) mutants followed by sequential reaction with alkaline-phosphatase conjugated streptavidin and pNPP as described.

5.3.10 ELISA to examine binding of gp41 3S motif to gC1qR

Microtitre wells were coated in duplicates with 100µl per well of 2µg/ml of gC1qR in carbonate buffer and incubated overnight at 4°C. The following morning the contents were discarded and wells were blocked with 5% non-fat milk in TBS for 1 hour at 37°C. Wells were then washed with TBS + 0.05% Tween 20 and different concentrations (1, 2.5 and 5µg/ml) of the gp41 3S motif peptide. The plate was incubated at 37°C for 1 hour and the wells were repeatedly washed. Bound protein was detected using affinity purified rabbit anti-3S IgG which was incubated for a further hour at 37°C. The secondary antibody used was alkaline phosphatase conjugated anti-goat and wells were developed using PNPP. Another identical ELISA was performed, with the exception of the proteins coated in the reverse order. Wells were first coated with 100µl per well of 100µg/well of the gp41 3S motif and different concentrations (1, 2.5, 5µg/well) of gC1qR were added. In this ELISA, bound gC1qR was detected with the pAb rabbit anti-gC1qR IgG.

5.4 Results

5.4.1 Location of deletions based on the crystal structure of gC1qR

After purity of the gC1qR deletion mutants was assessed by SDS-PAGE, it was determined to see whether the deletion of specific residues affects the ability of the protein to form a trimer (figure 5.5). This was assessed by gel filtration on a superose column. As shown in table, the deletion mutants 74-95, 204-218 and 212-223 were unable to maintain a trimeric structure. Crystal analysis reveals that the N-terminal residues 74-95 make up the α -helical coiled coil region of each monomer and plays a role in forming extensive intermolecular contact in maintaining the trimeric structure (Jiang et al, 1999, Ghebrehiwet et al, 2002). Residues 204-218 cover the β 6 structure of each monomer and residues 212-223 span the entire loop connecting β 6 and β 7 as well as covering the 20-A donut hole of the trimer (Jiang et al, 1999; Ghebrehiwet et al, 2002).

As the HCV core protein has been identified to bind to gC1qR, preliminary experiments were performed by a direct binding assay to confirm this interaction. The binding of gC1qR to the HCV core protein was represented in a dose dependent manner (figure 5.6).

Location of deleted residues and effect on trimerization

Deleted aa (Δ)	Position in 3D	Trimer Formation
WT gC1qR	NA	Yes
Δ 74-95	α A-coiled-coil, N-terminus	No
Δ 144-148	Loop β 3- β 4	Yes
Δ 144-162	Loop β 3- β 4	Yes
Δ 154-162	Loop β 3- β 4	Yes
Δ 174-180	Loop β 4- β 5*	Yes
Δ 190-202	Loop β 5- β 6	Yes
Δ 190-192	Loop β 5- β 6	Yes
Δ 196-202	Loop β 5- β 6	Yes
Δ 204-218	β 6+ loop β 6- β 7	No
Δ 212-223	Loop β 6- β 7*	No
W233G	α B, coiled-coil	Yes
* M-face residues		

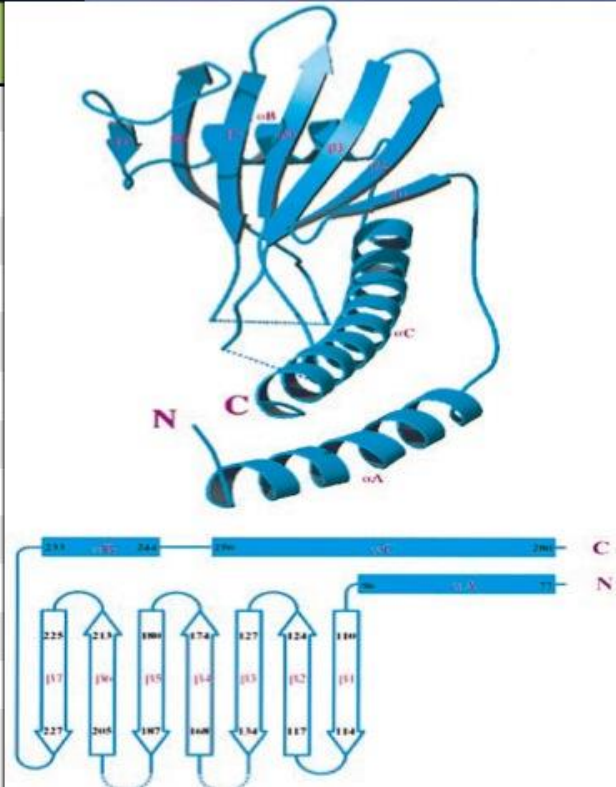


Figure 5.5. Location of deleted residues. The deleted residues and their effect on trimerization are as described (Ghebrehiwet et al., 2001) and are reproduced here together with the ribbon structure of a gC1qR monomer (Jiang et al., 1999) to show the position of the deleted residues.

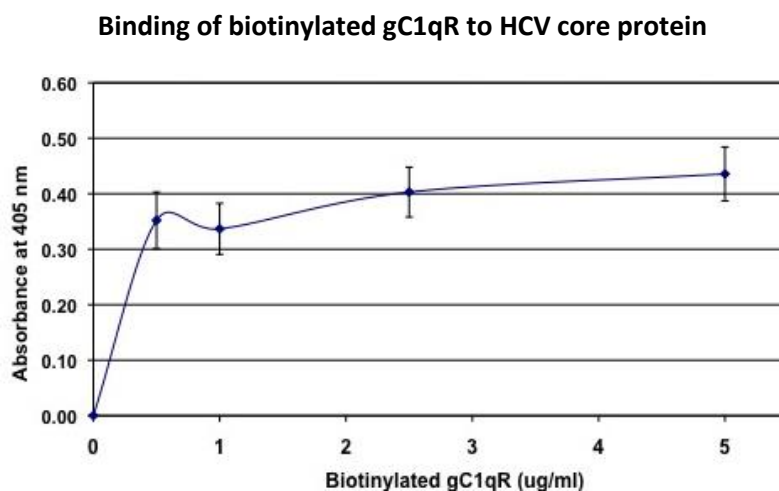


Figure 5.6. Dose-dependent binding of gC1qR to HCV core protein. Microtiter wells were first coated with 1 $\mu\text{g/ml}$ HCV core protein (1 h, 37 $^{\circ}$ C). After washing and blocking with 5% non-fat dry milk for 1 hour at 37 degrees, the bound HCV was reacted (overnight, 4 $^{\circ}$ C) with different concentrations of biotinylated WT gC1qR ranging from 0-5 $\mu\text{g/ml}$ to 5 $\mu\text{g/ml}$ followed by sequential reaction with alkaline-phosphatase conjugated streptavidin and pNPP following the standard procedure for ELISA. Each data point is a mean of duplicate experiments and the figure is a representative of three such experiments.

5.4.2 Identification of the gC1qR site for HCV core protein using gC1qR deletion mutants

As shown in Figure 5.6, the binding of HCV core to gC1qR is reproducible, specific, and dose-dependent. Previous studies (Kittlesen et al., 2000) have suggested that the binding site for HCV core protein resides on a long stretch of gC1qR that is encoded by exons IV-VI (residues 159-282). Therefore in order to verify and accurately define the HCV core protein interaction site on gC1qR, we used a solid-phase microtitre plate binding assay to test the interaction of a range of highly purified gC1qR deletion (Ghebrehiwet et al., 2001). When the binding of these deletion mutants was assessed using HCV coated-microtitre wells, two mutants lacking residues 144-148 and 196-202 respectively, showed significantly reduced interaction with HCV core protein when compared to the WT-gC1qR (Figure 5.7). Furthermore, the HCV core protein was found to bind both synthetic peptides (residues 144-148 and 196-202) but not to other peptides with similar charge and length (data not shown).

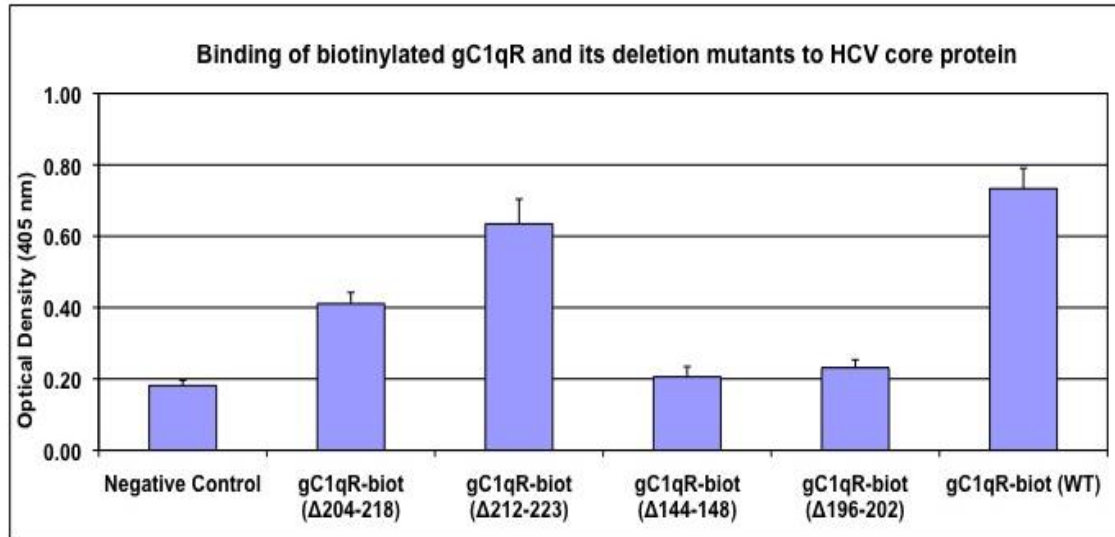


Figure 5.7. Interaction of gC1qR and gC1qR deletion mutants with HCV core protein. Microtiter plate wells were first coated with 1 $\mu\text{g}/\text{ml}$ of HCV core protein and incubated for 1 hour at 37 $^{\circ}$ C. Wells were washed three times with TBS + 0.05% tween and blocked with 5% non-fat dry milk for 1 hour at 37 $^{\circ}$ C. The bound HCV was reacted (O/N, 4 $^{\circ}$ C) with 5 $\mu\text{g}/\text{ml}$ of either biotinylated WT gC1qR or gC1qR deletion (Δ) mutants Δ 204-218, Δ 212-223, Δ 144-148 and Δ 196-202. The reaction was developed using alkaline phosphatase- streptavidin and PNPP. Data shown are representative of three separate experiments run in duplicates and includes data of only the deletion mutants that reacted weakly with HCV core protein.

5.4.3 Binding of gC1qR to gp41

When different concentrations of gC1qR were added to coated gp41, gC1qR bound gp41 in a dose dependant manner. The amount of bound gC1qR increased by 12% when the concentration was increased from 0.25 μg to 0.5 μg , and 28% when increased from 0.5 μg to 1 μg . BSA was used as a negative control. 1 μg is the optimum concentration for the binding of gC1qR to gp41.

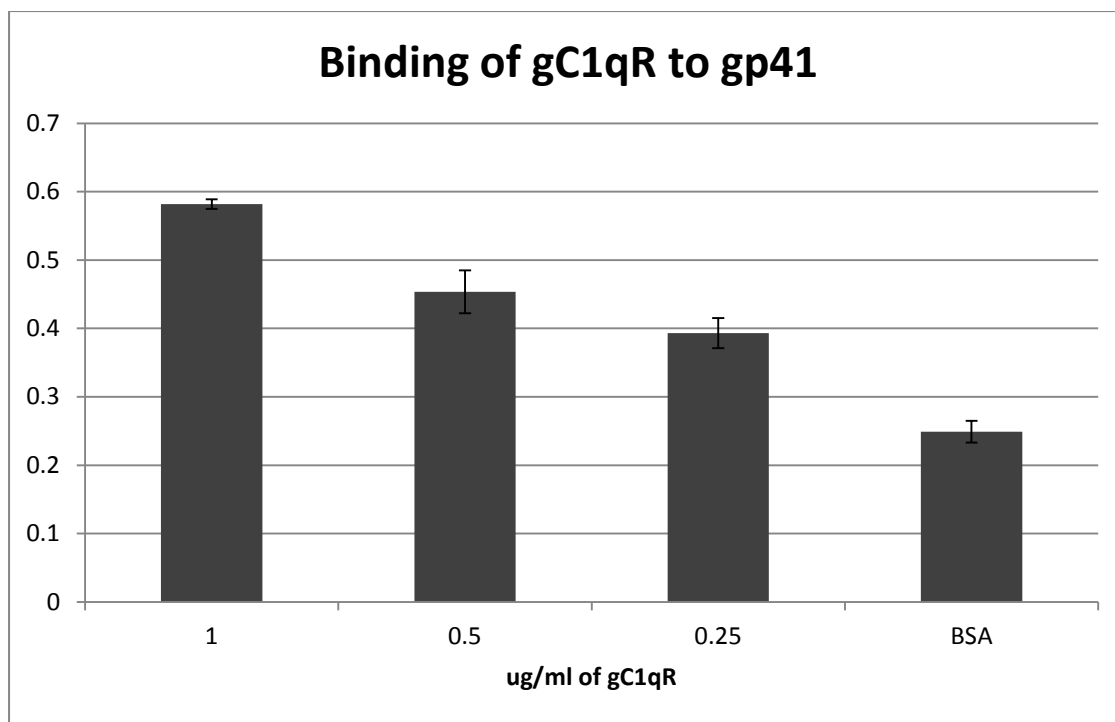


Figure 5.8. Binding of gC1qR to gp41: ELISA plate wells were first coated with 100 μ l per well of 2 μ g/ml of gp41 (1 h, 37 $^{\circ}$ C). Following blocking with 2% BSA for 1 h, wells were washed (3x with TBS-T) and incubated with different concentrations of gC1qR (1 μ g, 0.5 μ g and 0.25 μ g/ml) in TBS, (1 h, 37 $^{\circ}$ C). Bound gC1qR was then detected using mAb 74.5.2 anti-gC1qR (0.5 μ g/ml) followed by sequential reaction with alkaline phosphatase goat anti-mouse IgG and pNPP. The absorbance of the color developed after 30 min was measured spectrophotometrically at 405 nm. Each data bar is a mean of each experiment run in duplicates of which this is a representative of three such experiments.

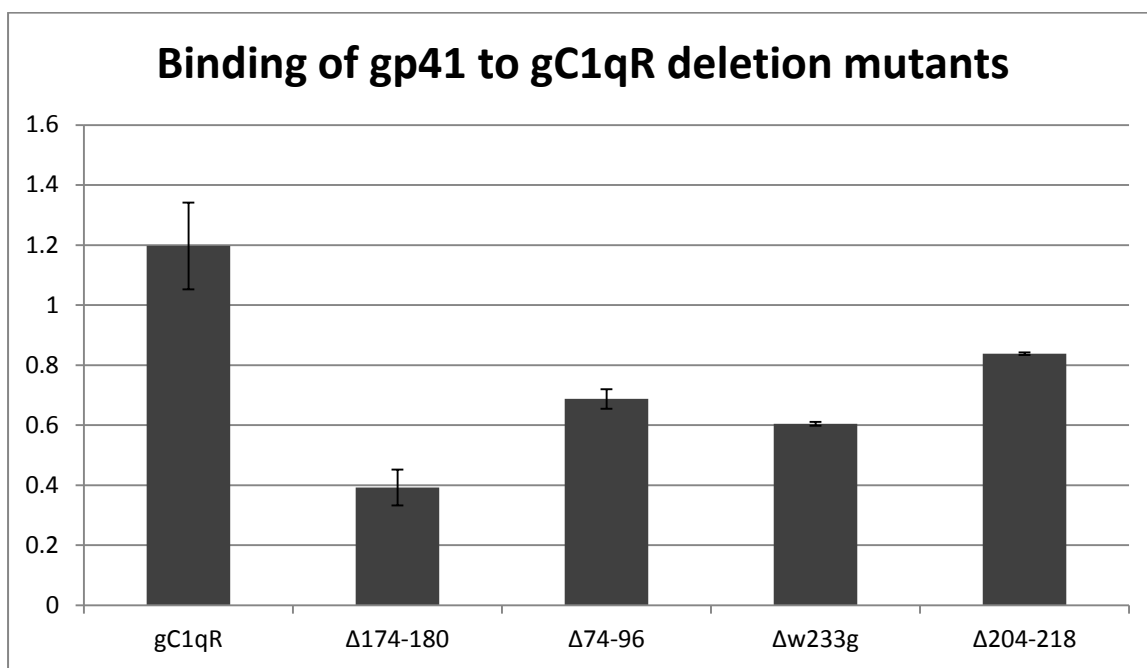


Figure 5.9. Identification of gp41 site on gC1qR: Microtiter plate wells were first coated with 1 $\mu\text{g}/\text{ml}$ HIV-gp41 (1 h, 37°C) and after washing and blocking with 5% non-fat dry milk, the bound gp41 was reacted (O/N, 4°C) with 2.5 $\mu\text{g}/\text{ml}$ of biotinylated WT gC1qR or gC1qR deletion (Δ) mutants and the reaction developed by standard ELISA procedure. Data shown are representative of three separate experiments run in duplicates.

5.4.4 Binding of gp41 to gC1qR deletion mutants

Since gC1qR does bind to gp41 in a dose dependant manner, we examined which residues on gC1qR are important for this interaction. As the 3S motif of gp41 located on residues 618 – 623 is identified as a gC1qR binder, we wanted to map the binding site on gC1qR that is complementary to this complex. Different concentrations of the deletion mutants of gC1qR, 174-180, 74-96, w233g and 204-218 were challenged with gp41 to assess binding. The deletion mutants of gC1qR were truncated due to the deletion of several residues from the full length sequence. The ELISA results showed binding was preferentially reduced when gp41 was challenged with the deletion mutant 174 – 180. Using WT gC1qR as a positive control, the results were compared against this point. gp41 bound 2 fold less to 174 – 180 in comparison to WT gC1qR. Deleting residues 204 – 218 reduced gp41 binding to gC1qR by 45% whereas deletion mutant 76-96 decreased binding to 75%. W233g is a truncated version of gC1qR with one amino acid deleted, Deletion of one amino acid abolished binding by one fold in

comparison to wild type gC1qR. Thus, residues 204-18 are not very critical in facilitating the interaction between gC1qR and gp41 as deleting these residues did not have as much of an effect as $\Delta 174-180$, 74-96 and w233g did. The finding that only the deletion of residues 174-180 in gC1qR reduced gp41 binding dramatically to 33%, suggests that these 6 residues are involved in this interaction with gp41. Interestingly, the deletion of 6 residues had greater effect than the deletion of 22 residues ($\Delta 74-96$) and 14 residues $\Delta 204 - 218$.

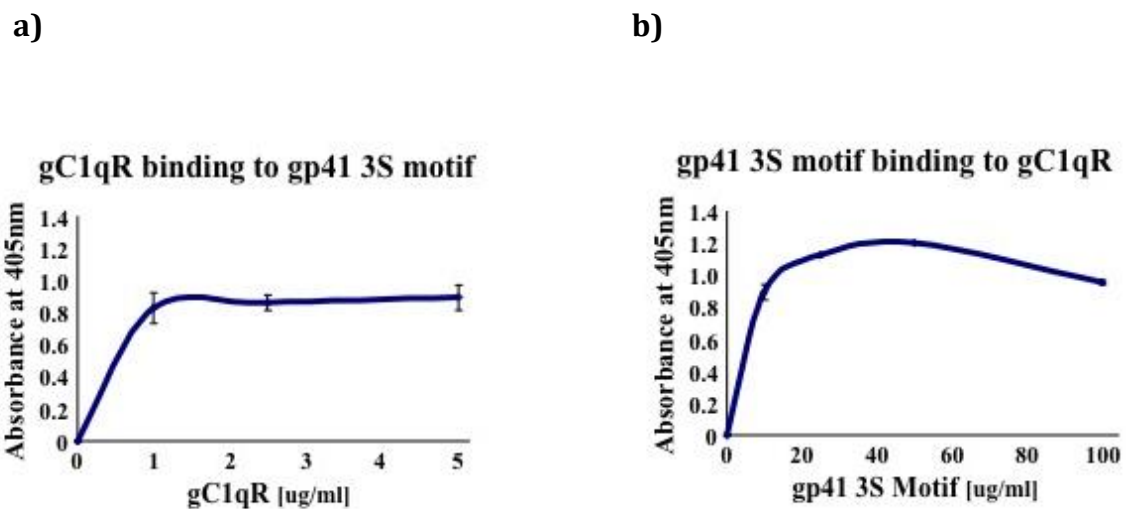


Figure 5.10. Interaction between HIV-1 gp41 3S motif and gC1qR. **a)** Duplicate wells of a microtiter plate were coated with 100 μl of 2 $\mu\text{g/ml}$ gC1qR and incubated (O/N, 4 ° C). Then, after blocking the unreacted sites with 5% non-fat dry milk, the bound gC1qR was reacted (1 h, 37 ° C) with concentrations of synthetic gp41 3S peptide and the bound peptide detected using an affinity purified rabbit anti- S IgG. In **b)** the reaction sequence was reversed in that the wells were first coated with 100 μl of 100 $\mu\text{g/ml}$ 3S peptide, reacted with increasing concentrations of gC1qR and the bound gC1qR detected using rabbit anti-gC1qR IgG.

5.4.5 Identification of the gC1qR site for the HIV-1 gp41

Similarly, the binding of gC1qR to gp41 coated microtitre wells is also dose-dependent Figure 5.8. Subsequent binding assays comparing WT-gC1qR and its deletion mutants showed (figure 5.9) that only gC1qR $\Delta 174-180$ had a significantly reduced binding to gp41 (n=3). Furthermore, since the 3S motif of gp41 (residues 601-620) has been reported to be the binding site for gC1qR (Fausther-Bovendo et al., 2010), we generated

a synthetic peptide spanning the putative 3S residues (PWNASWSNKSLDDIW), and tested the ability of this peptide to bind to gC1qR coated wells. As shown in figure 5.10 there was a strong and dose-dependent interaction over a wide-range of concentrations. Conversely, gC1qR was also able to bind to 3S-coated plates, which was inhibited by anti-3S antibody (data not shown).

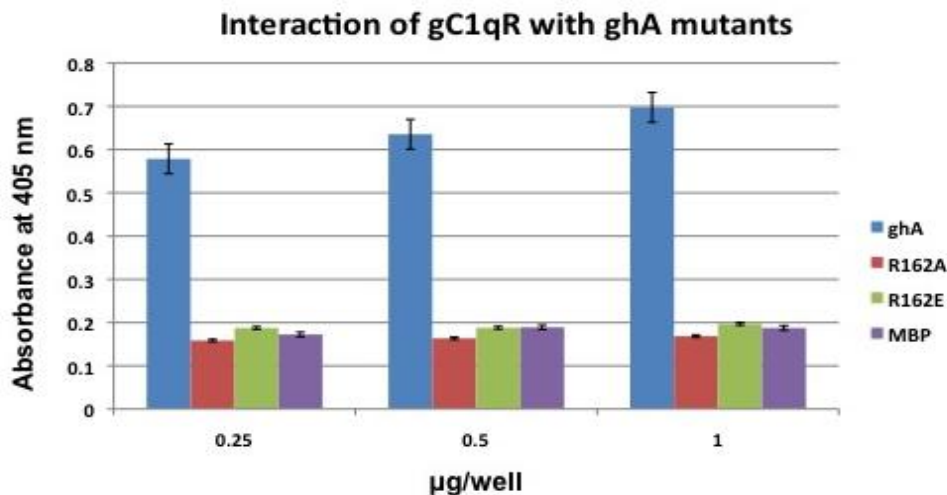


Figure 5.11: Interaction of ghA mutants with gC1qR: Microtiter wells were coated with 1.0, 0.5 or 0.25 µg/well of gC1qR in carbonate buffer and the plate incubated at 4° C overnight. The next morning, contents were discarded and wells were blocked for 2 h with 2% BSA at 37° C. After washing with PBS + 0.05% Tween, 2.5 µg/well of ghA wild type, R162A, R162E and MBP was added and the plate was incubated 1.5 h, 37° C and 1.5 h at 4° C. Wells then were washed and anti-MBP (1/5000) was added, incubated for 1 h 37° C and the bound protein was detected using horseradish peroxidase conjugated IgG. After addition of OPD buffer, the colour developed was read at 450 nm.

5.4.6 The ghA domain of C1q is the binding site for both gC1qR and gp41

Previous studies using both ligand blot and peptide mapping analyses have shown that gC1qR binds to gC1q predominantly via the A chain (ghA residues 155-164) and that the two adjacent Arg residues at positions 162 and 163 are critical for this binding (Ghebrehiwet et al., 2001). This conclusion was further substantiated by the finding that gC1qR binding to this A chain peptide was abolished when the two Arg residues were substituted with Gln residues (Ghebrehiwet et al., 2001). In the present study, the

binding of gC1qR to recombinant ghA module was compared to ghA mutants in which the Arg at position 162 was substituted with either Ala (R162A) or Glu (R162E). As shown in figure 5.11 while gC1qR binds well to recombinant ghA, there was no binding with either ghA R162A or R162E. Similarly, the HIV-1 gp41 glycoprotein also binds to C1q to the same domain in the A-chain of gC1q (data not shown).

Potential recruitment of C1q and gp41 to gC1qR on T cells during HIV Infection

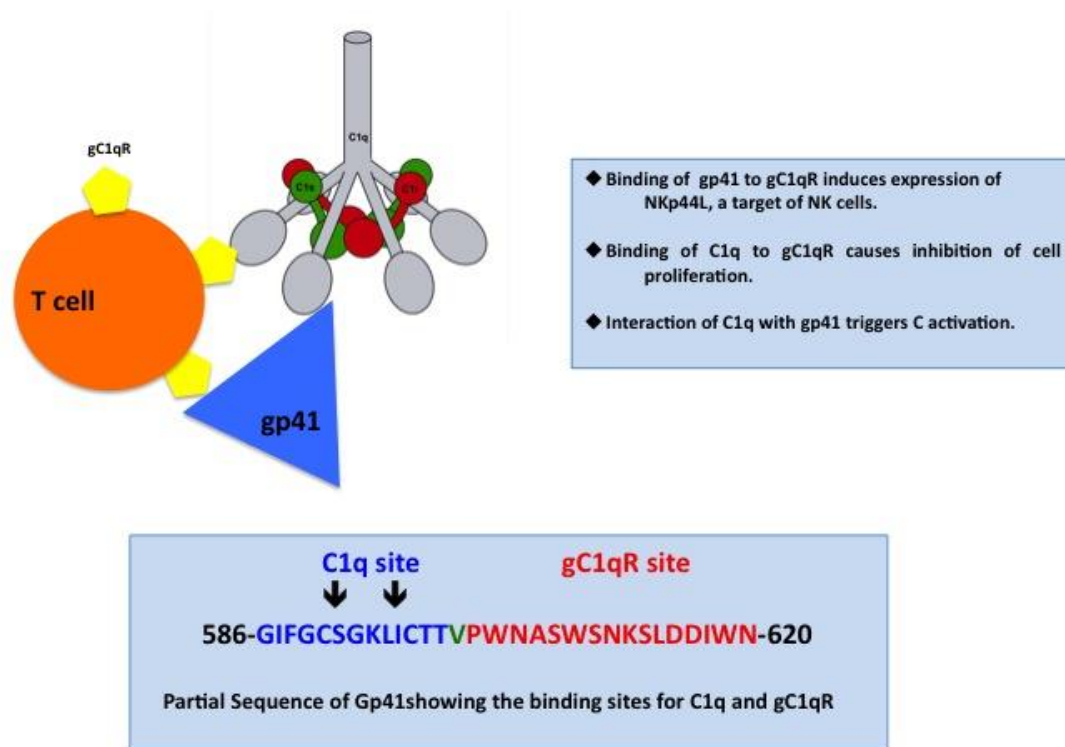
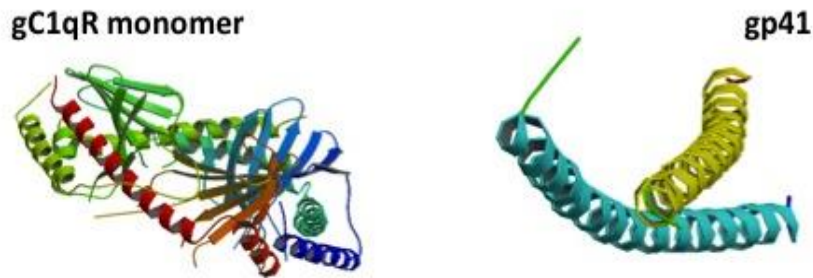


Figure 5.12. The 3S motif carries the C1q and gC1qR binding sites. Schematic representation of the interaction between gC1qR, the 3S domain of HIV-1 gp41, and complement protein C1q.

Structural alignment of gC1qR monomer and gp41



249:C	260:C	270:C	280:C
.			
DNTFADELVELSTALEHQEYITFLEDLKSFVKSQ			
..... : ...::...			
IVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL			
.			
548:N	560:N	570:N	580:N

... Structurally equivalent and identical residues
: ... Structurally equivalent and similar residues
. ... Structurally equivalent, but not similar residues.

Dimensional alignment showing structural similarity between gC1qR and gp41.

Figure 5.13. gC1qR shares 3-D structural similarity with gp41. Dimensional alignment of gp41 and gC1qR reveals that gC1qR shares a 3-D structural similarity with transmembrane protein gp41, with a p-value of 8.72E-6. One monomer of gC1qR (C chain, 176 residues) is aligned with chain N (gp41, 51 res).

5.5 Discussion

As discussed earlier (Ghebrehiwet et al., 2001), the gC1qR molecule is targeted by a wide range of pathogenic microorganisms and as such is considered to be an emerging receptor for pathogen-associated molecular ligands. Among these important pathogens are: HCV and HIV-1, both of which suppress T cells via gC1qR (Yao et al 2004). HCV is a major cause of chronic hepatitis worldwide, and is the leading cause of hepatocellular carcinoma, and to date there is no effective treatment (Cummings et al., 2009). Similarly, the mortality and morbidity caused by HIV infection is staggering, with more than 34 million people infected presently and over a million-and half deaths worldwide in 2011 alone. Therefore, understanding at the molecular level, the virus-host cell interaction and, in particular, identification of novel molecular targets by which these pathogens cause pathology will not only give us insight into how they escape the onslaught of the innate immune defense mechanism, but may provide us with alternative approaches for designing better therapeutic targets

Previous studies have shown that the 22 KDa non-glycosylated HCV core protein binds gC1qR on CD4⁺ T cells and induces an anti-proliferative response (Kittlesen et al., 2000). Suppression of T cells is considered to contribute to an extremely high rate of viral persistence and severe disease progression. Although the binding of HCV core protein has been identified to be in a domain within a long stretch of gC1qR comprising of residues 188-259, the precise binding site has not been identified. In this study, we not only confirmed that the interaction site is within this segment but also, using several gC1qR deletion mutants refined and mapped the HCV core protein site(s) on two gC1qR domains comprising of residues 144-148 and 196-202. These residues make up the loops in each monomer that connect $\beta 3$ to $\beta 4$ and $\beta 5$ to $\beta 6$, respectively, in the structure of the protein (figure 5.5). These prominent loops, which cover up much of the doughnut hole of the gC1qR homotrimer, are highly charged and probably form a binding cluster in space. Interestingly, the doughnut hole covered by these residues also overlaps with the binding site for HK (Ghebrehiwet et al., 2002).

Similarly, the envelope glycoprotein gp41 of HIV-1 interacts with gC1qR on CD4⁺ T cells. This interaction is considered to enhance the destruction of uninfected T cells by activated NK cells thereby contributing to the persistently low level of T cell count seen

in the late phase of the disease (Fausther-Bovendo et al., 2010). While the gC1qR interaction site on gp41 has been mapped to the 3S motif and comprises of residues 601-620 (Fausther-Bovendo et al., 2010), the complementary binding site for gp41 on gC1qR has not been identified.

After preliminary experiments confirmed that gC1qR does bind to gp41 in a dose dependant manner, we were able to use several deletion mutants of gC1qR (74 – 86, 174 – 180, w233g and 204 – 218) and challenge them with gp41 to observe for changes in affinity binding.

Here, we have identified gC1qR residues 174-180 as the major binding site for HIV-1 gp41. Domain 174-180, which has recently been shown to contain the cell surface-binding site for soluble gC1qR, is a highly conserved loop, which connects β 4 to β 5 in the 3D structure of gC1qR (Figure 5.5). Previous studies by others have also shown that C1q, the natural ligand for gC1qR, binds to gp41 and the binding site was mapped to a complement site intact on a domain (residues 586-598), which is adjacent to the 3S motif of gp41 residues 601-620) (Thielens et al, 1993; Quinkal et al, 1999). Although the biological significance of this remains to be assessed, it is intriguing to find that the binding sites for both gC1qR and C1q are localized in the same stretch of the gp41 3S domain, and may therefore overlap (Figure 5.2). The biological implications of the simultaneous binding of gp41 and C1q to CD4⁺ T cells remain to be ascertained. Viral envelope proteins such the outer envelope protein gp120 of HIV-1 share certain structural and functional similarities with C1q (Szabo et al., 2001). Three -dimensional alignment (Fig 5.13) of the gC1qR monomer with gp41, for example, reveals structural similarity between the two proteins. Therefore, it was predicted that proteins like gC1qR that are able to interact with C1q may also interact with isolated gp120 or even with the whole HIV-1 virus. Thus, gC1qR has been shown to effectively and dose-dependently inhibit the production of one T-lymphotropic (X4) and one macrophage-tropic (R5) strain in human T cell lines (MT-4 and H9) and human monocyte-derived macrophage cultures, respectively (Szabo et al., 2001). Interestingly, the extent of this inhibition was enhanced when gC1qR was first incubated with, and then removed from the target cell cultures before virus infection, compared to when the cells were infected with gC1qR-HIV mixtures (Szabo et al., 2001). Furthermore, these experiments showed

that although gC1qR did not bind to a solid-phase recombinant gp120, it showed a strong and dose-dependent binding of gC1qR to solid-phase CD4 (Szabo et al., 2001). Since gp120 within the gp120/gp41 complex determines viral tropism and the role of gp41 is to mediate fusion between viral and cellular membranes (Caffrey, 2001), gC1qR may prevent the latter process by binding to gp41 within the complex in the early phase of viral infection. While these results imply a beneficial role of gC1qR for the host, a darker side to this interaction is when gp41, potentially released during infection, interacts with cell surface gC1qR to favor viral survival through destruction of CD4⁺ cells (Stocker et al., 2000) (Vieillard et al., 2005). Since the great majority of dying cells during the later phase of HIV-1 infection are the uninfected CD4⁺ T cells, the interaction of gp41 and gC1qR can play a significant role in perpetuating the disease process by enhancing the autologous destruction of these cells (Fausther-Bovendo et al., 2010, Vieillard et al., 2005). The destruction of uninfected T cells is, therefore, postulated to be in part, responsible for the persistently low T cell count during the later stages of viral infection

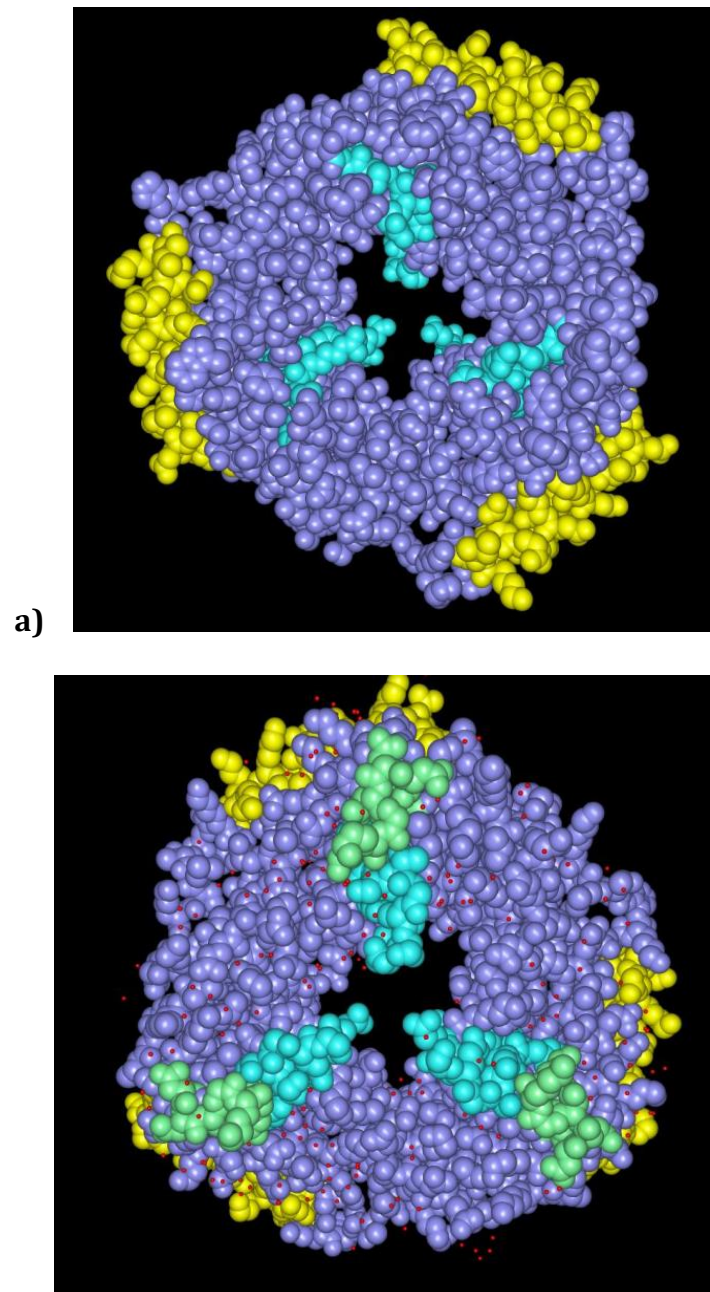


Figure 5.14: Structural image to highlight the structure of gC1qR indicating hypothesised gp41 binding region: a) gC1qR in its solution face b) gC1qR in its membrane face. This structural image was constructed to highlight and visualise the suggested gp41 binding site which is shown in blue. The green regions represent the binding site for HK corresponding to regions 204-219 and yellow regions for C1q binding site being 74-96.

The identification of gC1qR domain 174-180 as the major binding site for gp41, therefore presents an alternative opportunity for the design of either peptide-based or antibody-based therapy that salvages CD4⁺ T cells from gp41 mediated destruction. This postulate is further substantiated by the fact that a synthetic peptide corresponding to domain 174-180 is able to block the interaction between gp41 and gC1qR. In addition to this, residues 174 – 180 appears to be a highly conserved sequence evident in a range of homologues, as shown in the table below

gC1qR peptide MF-1 (aa 174-180)	I K N D D G K K A
<i>Trichomonas vaginalis</i> G3	I K N D D <u>L</u> K K A
<i>Borrelia valaisiana</i>	I K N D D G K K <u>I</u>
<i>Borrelia burgdorferi</i> B31	I K N E D G K K V
[Translation initiation factor (IF-2)]	I K N D D G <u>R</u> K A
Zebrafish	

Table 5.1: List of homologues that contain the sequence 174-180: A range of species share the highly conserved sequence 174-180 found in gC1qR

The fact that such a wide range of organisms hold such a similar sequence, and present data supports the identification of this motif as a binding site, makes it extremely puzzling as to what physiological relevance is held within such a sequence.

CHAPTER 6:

**Endothelial Cell gC1qR and its effect on
vascular permeability via bradykinin
receptor 1 (B1R)**

6.1 Abstract

The endothelial cell (EC) receptor complex for high molecular weight kininogen (HK) involves two-component system: cytokeratin 1-uPAR (urokinase-type plasminogen activator receptor), and gC1qR-cytokeratin 1, required for the assembly and activation of the coagulation/KKS. FXII preferentially binds to the cytokeratin 1-uPAR complex while gC1qR is a high affinity receptor for HK and contributes to KKS activation leading to the generation of BK. BK, a potent vasodilator agonist, belongs to the kinin family of proinflammatory peptides, which induce their activity via two G-protein-coupled receptors: bradykinin receptor 1 (B1R) and B2R. B2R is constitutively expressed on a range of normal cells including ECs. However, B1R is induced and expressed by IL-1 β . We hypothesized that soluble gC1qR (sgC1qR) can serve as an autocrine/paracrine signal for the induction of B1R expression based on the evidence that (1) gC1qR is secreted by activated ECs at sites of inflammation, and (2) sgC1qR can bind to cells in a specific and dose-dependent manner. In this chapter it is being shown that (1) gC1qR binds to microvascular ECs predominantly via a gC1qR domain (residues 174-180); (2) sgC1qR induced enhancement of B1R expression by ECs dose-dependently; (3) The effect of gC1qR on B1R expression was greatly diminished by gC1qR lacking residues 174-180 (gC1qR Δ 174-180) and by gC1qR Δ 154-162. Furthermore, (4) a synthetic peptide comprising of residues 174-180 mimicked the effects of the parent molecule. Although ECs normally express detectable amount of B1R, this base level expression increases slightly in the presence of gC1qR even after 2 hr incubation, indicating translocation of stored B1R. Finally, the binding of soluble gC1qR was predominantly through surface-expressed fibrinogen, which is inhibited by anti-fibrinogen antibodies. Thus, at sites of activation and inflammation, ECs secrete soluble gC1qR, which binds to a fibrinogen-like surface molecule. This interaction can enhance vascular permeability through upregulation of B1R *de novo* synthesis as well as rapid translocation of preformed B1R.

6.2 Introduction

The EC receptor for HK involves two complexes: uPAR-cytokeratin 1 and gC1qR-cytokeratin 1 (Joseph et al., 1996, Herwald et al., 1996, Hasan et al., 1998, Colman et al., 1997, Pixley et al., 2011, Mahdi et al., 2001, Joseph et al., 2004). gC1qR serves as a zinc-dependent high affinity receptor for HK. It is critical for the assembly and activation of the plasma KKS system leading to the generation of BK (Joseph et al., 1996, Pixley et al., 2011). The KKS consists of three components (Kaplan, 2002): coagulation factor XII [FXII (or Hageman Factor, HF)], prekallikrein (PK), and HK. HK is known to release TNF- α , IL-1 β , IL-6 and IL-4 from human mononuclear cells (Khan et al., 2006). While FXII preferentially binds to the uPAR-cytokeratin 1, HK, which circulates in complex with PK, binds to gC1qR through residues located in its domain 5 (Kaplan, 2002). Once factor XII is activated to factor XIIa, it converts prekallikrein to kallikrein, a process that is enhanced by heat shock protein 90 and/or the enzyme carboxypeptidase released by ECs (Joseph and Kaplan, 2005). Kallikrein digests HK to generate the first kinin produced from kininogens, the nonapeptide BK (NH₂-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) (Kaplan, 2002). Kinins are potent vasoactive pro-inflammatory peptides that are generated in response to pathological stimuli by their kininogen precursors (Bhoola et al., 1992). BK belongs to the kinin family of proinflammatory peptides and is among the most potent vasodilator agonists known (Regoli and Barabe, 1980, Bhoola et al., 1992). Once generated, it is rapidly converted to des-Arg⁹-BK via the removal of the C-terminal arginine. BK induces its activity via two G-protein-coupled receptor subtypes: bradykinin receptor 2 (B2R) and to a lesser extent, B1R. These kinin receptors are expressed on both peripheral and central ECs, where they couple to the G proteins G_q and G_i to induce an increase levels of intracellular Ca² and nitric oxide, which promotes vascular permeability and vasodilation.

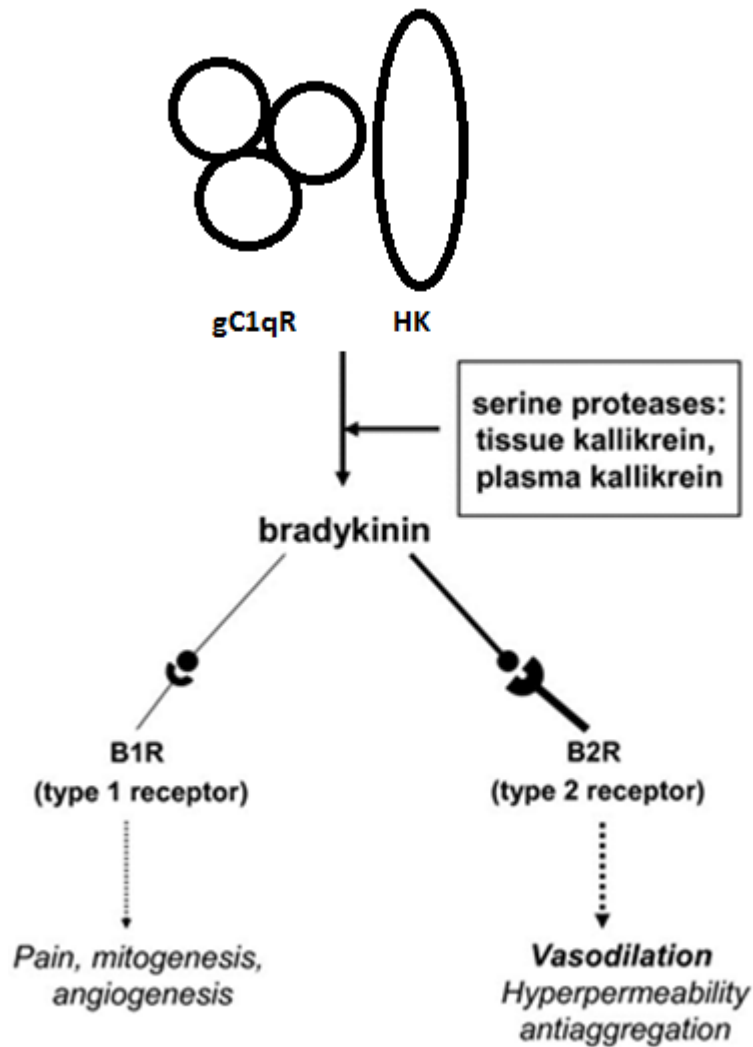


Figure 6.1: The Kinin System: HK is able to bind to gC1qR and activate the vasoactive peptide BK causing the vessel wall to become more permeable, leading to an influx of tissue fluid causing inflammation (Valdes et al., 2009).

While B2R is constitutively expressed on a number of normal cells including ECs, B1R is expressed at low levels but induced rapidly following tissue damage by IL-1 β and responds to the carboxypeptidase product desArg⁹-BK (Leeb-Lundberg et al., 2001). BK has a higher affinity for B2R, des-Arg⁹-BK is a selective ligand for B1R. This BK receptor activation of both receptors is known to contribute to a number of acute and chronic diseases including hypotension, bronchoconstriction, pain and inflammation (Menke et al., 1994; Hess et al., 1992)

During the inflammatory response, activated cells including ECs are known to overexpress and secrete soluble gC1qR (sgC1qR). Soluble gC1qR can bind to intact cells in an autocrine/paracrine manner (Peterson et al., 1997). gC1qR can bind HK on ECs activate the kinin pathway (Kaplan et al., 2001). Ghebrehiwet et al (2013) have thus identified additional HK binding sites on gC1qR using gC1qR deletion mutants. In solution, BK is generated when mixed with HK, PK and FXII and incubated for hours. However, with the addition of gC1qR, this process can be completed within minutes. Thus, BK can be generated in the presence of soluble gC1qR. Therefore, once gC1qR is secreted into the pericellular milieu by activating cells, gC1qR can potentially bring upon BK generation in the presence of circulating PK, HK and FXII (Kaplan et al., 2001). Soluble gC1qR from culture supernatants of proliferating cells binds to U937 and ECs. Blocking gC1qR and B1R inhibits BK-induced angioedema (Bossi et al., 2009), which suggests that gC1qR could potentially behave as an autocrine signal to induce B1R expression. Therefore, blocking gC1qR could prove helpful in inhibiting the generation of BK, pointing a way forward to peptide based therapies to control vascular permeability and inflammation. Here, we show that gC1qR upregulates B1R on ECs via its interaction with surface expressed fibrinogen.

6.3 Materials and Methods

6.3.1 Chemicals and reagents

The following reagents and chemicals were purchased or obtained from the sources indicated: D-PBS with and without calcium and magnesium (Mediatech Inc, Manassas, VA); Dulbecco's Modified Eagles Medium (DMEM); RPMI 1640, 100x Penicillin/Streptomycin, (GIBCO-Invitrogen); heat inactivated fetal bovine serum (FBS) (Hyclone); human serum albumin (HSA) (Immuno-US); *p*-nitrophenyl phosphate (pNPP) (Pierce); and Immu-Mount (Thermo Fisher). Alexa 488- or Alexa 594-Streptavidin, Alexa 488- or Alexa 594-F(ab')₂, goat anti mouse or anti rabbit; FITC conjugated goat anti-mouse IgG F(ab')₂ or sheep anti-rabbit IgG F(ab')₂ (Invitrogen); and alkaline phosphatase (AP)-conjugated rabbit anti-goat IgG (Pierce), and C4d-EIA test kit (Quidel).

6.3.2 Expression of recombinant gC1qR and its deletion mutants

The strategy for the construction of plasmids containing the full length as well deletion mutants lacking highly charged domains has been described in Chapter 5.

6.3.3 Cultured cells

Human brain microvascular endothelial cells (HB-MVECs) were cultured in EC Growth Medium-2 (EGM-2MV; Lonza,) containing 5% heat inactivated FBS as described previously (Yin et al., 2007). Cells grown to confluence on 2 % gelatin were first treated with 0.25 % trypsin, 0.01 % EDTA to dissociate cells from the gelatin matrix by incubation (30 min, 37^o C) in 0.01 M Tris-buffered saline. The cells were then washed and sub-cultured in Dulbecco's Modified Eagles Medium. Experiments were done with cells between passages 3-15. The U937 cell line was grown in suspension in RPMI1640 containing 10% heat inactivated fetal bovine serum and 100 units/ml penicillin and 100µg/ml streptomycin and maintained in a humidified air consisting of 5% CO₂ and 95% air as described (Randazzo et al., 1988). Prior to each experiment, the viability of cells was verified by trypan blue exclusion.

6.3.4 Proteins and antibodies:

Fibrinogen and rabbit anti-fibrinogen IgG were a generous gift from Dr. Dennis Galanakis (Stony Brook University). Purified rabbit IgG antibodies to bradykinin receptor 1 (B1R) and B2R were purchased from Abcam Inc. (Cambridge, MA) and affinity purified rabbit IgG (designated pAb UN-15) to a synthetic gC1qR peptide was generated commercially (GenScript, Piscataway, NJ).

6.3.5 SDS-PAGE and Western blot analysis

Analyses on SDS-PAGE was performed on a 1.5 mm thick slab gels according to the method of Laemmli (Laemmli et al 1970) with samples being run unreduced or reduced and alkylated by boiling for 5 min in the presence of 0.1 M dithiothreitol and 0.2 M iodoacetamide. After electrophoresis, the gels were stained either with Coomassie Brilliant Blue, or silver stain, then destained and dried. Samples for Western blot analysis were first run on SDS-PAGE as above, then electrotransferred to PVDF nitrocellulose membranes, blocked with 5% non-fat milk containing Tris Base Saline

(TBS)-T (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20), and the bound proteins probed with the appropriate and specific antibodies. The bound antibodies in turn were visualized by chemiluminescence horseradish peroxidase-conjugated species-specific antibody followed by reaction with 4-chloro-1-naphthol substrate.

6.3.6 Biotinylation of U937 cells

A total culture of 125ml of U937 cells grown in FBS were used (25ml in 5 tubes), which corresponded to 125×10^8 . The cells were first checked for viability. This involved adding 10 μ l of trypan blue to 1ml of cells and observing the cells under a microscope. Once the cells were verified for viability, the cells were spun down at 1400rpm for 8 minutes. The supernatant was discarded and 3ml of DPBS was added to each pellet in each tube. The pellets were resuspended and pooled together and further DPBS was added to bring the volume up to 50ml. This mixture was then spun down at 1400rpm for 8 minutes. Once again, the DPBS was discarded and the pellet was resuspended in 5ml DPBS and 100 μ l of NHS-LC-Biotin mix in DMSO at a concentration of 3mg/ml was added to the pellet mixture and left on a tumbler in the cold room for 2 hours. After this incubation, the mixture was spun down at 1400rpm for 5 minutes. The supernatant was discarded and 4ml of solubilisation buffer was added and the tube was left on ice for 1 hour. Following this, the mixture was centrifuged at 1400rpm for 15 minutes and the supernatant was collected and transferred to separate eppendorf tubes. These tubes were spun down in a high speed centrifuge for 30 minutes and the supernatant was collected, aliquoted and stored at -20.

6.3.7 Identification of membrane proteins by antigen capture assay

U937 cells were first grown in either RPMI 1640 with heat inactivated FBS or serum-free (without FBS). After the cells reached confluence ($\sim 1 \times 10^6$ /ml), the contents of each culture flask (100 ml) were separately washed 3 x by centrifugation (800g, 4 $^{\circ}$ C, 10 min). Then, each cell pellet was re-suspended in 5 ml of 5 mM DPBS, pH 7.5 containing 0.5 mM EDTA, 10 mM EACA, and 0.5 mM phenyl methyl-sulfonyl fluoride (PMSF). Cell membranes were then prepared by freeze thawing (five times) in liquid nitrogen followed by centrifugation (1 h, 45.000g, 4 $^{\circ}$ C). The supernatant containing the cytosolic fraction was removed and the pellet containing the cell membrane was used to prepare membrane proteins by solubilization in DPBS containing 1% NP-40. The solubilized

membrane proteins were freed from insoluble material by centrifugation (1 h, 30,000g, 4° C) after which the total protein concentration was determined by the detergent compatible Cultures with ≥95% viability were used for experiments.

6.3.8 Protein Capture Assay

Microtitre wells were coated with 100µg/well in coating buffer, pH 9.6 of the gC1qR peptide 174-180. As a positive control, the wells of gC1qR were coated at a concentration of 2µg/ml. This was coated to clarify that any protein(s) observed to bind to the peptide sequence of gC1qR would clearly have to bind to the full length protein itself if this binding is true. Once the plate was coated, it was incubated at 37°C for 2 hours to ensure thorough binding. After this, the contents were discarded and the wells were blocked with 3% v/w Non-fat milk powder in TBS for 1 hour at 37°C. After this the wells were washed 3 x in TBS-T and 100µl of biotinylated and solubilised U937 membrane lysates were added to each well. This was left at 37°C for 2 hours following overnight incubation at 4°C.

6.3.9 Protein Precipitation

Following overnight incubation with the U937 cell lysate, the wells were washed 3x with TBS-T and 100µl of glycine pH 2.6 was added to each well and left at room temperature in a shaker for 30'–1 hour. While this incubation was taking place, the required volume of Acetone needed was frozen. 1ml of each protein sample was to be pooled from the wells, thus 2 tubes with 4ml of acetone were labelled '174-180 peptide' and 'gC1qR', and were both frozen at -20 for 30'–1 hour. Once the glycine incubation was over, each 100µl of glycine from the 174-180 peptide coated wells were pipetted out and pooled together in an eppendorf tube to give a total sample volume of 1ml (10 wells at 100µl each). Whilst pipetting was performed to remove the glycine, the pipette tip was scraped against the well to ensure removal of all bound protein. The same procedure was performed for the gC1qR sample. Next, the cooled acetone was removed from the freezer and 1ml of each protein sample dissolved in glycine was added to their respective acetone tubes to give a new volume of 5ml. The tubes were then incubated at -20°C for up to 2 hours to allow complete precipitation. Next, the samples were centrifuged at 14,000xg for 10 minutes. The supernatant was discarded carefully

without disturbing the pellet. Next, the tubes were left uncapped at room temperature to allow any remaining acetone to evaporate.

6.3.10 Silver Staining

50µl of sample buffer was added to the pellet and resuspended and the samples were boiled at 100°C for 10 minutes. After spinning, the 50µl samples were loaded in a 12% SDS gel and 1µl of Biorad precision marker as standard. Silver staining method was used to visualise the proteins. The gel was placed in a clean plastic dish and washed in a generous amount of ultrapure dH₂O 2 times for 5 minutes each. Next, the gel was washed 2 x for 15 minutes in fixing solution (30% ethanol and 10% acetic acid). The gel was then washed 2 x for 5 minutes each in 10% ethanol. Following this, the gel was washed 2 x for 5 minutes in dH₂O. During this incubation, the sensitizer working solution was prepared by mixing 1 part Silver Stain Sensitizer with 500 parts dH₂O, (50µl Sensitizer with 25ml water). The gel was then sensitized for 1 minute and then washed 2 x for 1 minute each with dH₂O. During this incubation the Stain Working Solution was prepared by mixing 1 part Silver Stain Enhancer with 50 parts Silver Stain, (500µl Enhancer with 25ml Stain). The gel was incubated in this Stain Working Solution for 30 minutes. While the gel was being stained, the developer working solution was made by mixing 1 part Silver Stain Enhancer with 50 parts Silver Stain Developer, (500µl of Enhancer with 25ml Developer). Once the 30 minutes of staining was over, the gel was washed 2 x for 20 seconds each with dH₂O. Next, the developer working solution was added and the gel was developed for 2-3 minutes until the bands appeared. Once the desired band intensity was reached, the developer working solution was discarded and the stop solution, (5% acetic acid) was added to the gel for 10 minutes. Following this, the gel was washed briefly in water and covered in plastic. .

6.3.11 Protein capture assay of solubilised membranes of U937 cells grown in serum free media

U937 cells grown in serum free media this time were solubilised to form membrane lysates following the solubilising steps as described. Following this, capture assay as mentioned in figure 6.7 was performed, along with the protein precipitation and Silver Staining procedure.

6.3.12 Western Blot of 174-180 peptide pull down assay from biotinylated, solubilised U937 membrane lysates grown in serum free media

A 12% SDS-PAGE was prepared using the biorad rainbow marker. 50µl Sample buffer was added to the protein pellet and the sample was heated at 100°C for 10 minutes. Following a quick centrifuge the whole sample was loaded into the well and the gel was run at 120V and transferred onto PDVF membrane

6.3.13 Western blot detection using Chemi-immunofluorescence

Once the transfer was finished, the nitrocellulose membrane was carefully placed into a plastic cassette using tweezers and blocked for 1 hour at room temperature in blocking solution made up of 3% Non-fat milk powder in PBS. Following this, the membrane was incubated in HRP-Streptavidin at a concentration of 1:1000 in 1ml of 3% milk powder. This incubation was performed overnight at 4°C. The next morning the membrane was washed 5 x in TBS-T for 20 minutes each to ensure removal of any background. Next the membrane was placed in an open plastic wallet and sprayed with HyGLO Quick Spray which is a mixture of two reagents (Luminol enhancer and peroxide buffer) which allows a Chemiluminescence signal on HRP conjugated antibodies. The membrane immersed in the mixture was covered with the plastic wallet, and kept on for 1 minute. Following this, the excess substrate was blotted out with a paper towel and the membrane was transferred using tweezers to a fresh plastic wallet, which was taped to the Western Blotting Cassette and closed. The cassette and the Kodak films, which the bands would be lit up on, were taken to the dark room for developing. Once in the dark room, any handling of the films was carried out in darkness, the cassette was opened and the film was carefully placed on top. The first exposure was performed for 10 seconds and the film was carefully removed and processed through the machine to visualise the transferred protein bands.

6.3.14 Flow cytometry

U937 cells were collected following trypsinisation, and washed in PBS-A buffer (PBS containing 1% BSA and 0.01% NaN₃). Non-specific binding was blocked with 1 mg/ml human IgG or Fc fragments in PBS-A per 1x10⁶ cells (30 min, 4°C) and primary Abs or the appropriate isotype-matched controls were added to the cells (30 min, 4°C). Cells were washed twice in PBS-A, and further incubated with FITC-labeled secondary Abs

(30 min, 4°C). The cells were washed in PBS-A, fixed in 10% formalin and analyzed by flow cytometry using FACSCalibur (Becton-Dickinson, Mountain View, CA). For each analysis, 5,000 events were collected. The data was analyzed using CellQuest Pro software (Beckton-Dickinson, BD).

6.3.15 immunofluorescent microscopy

Immunofluorescence studies were performed on either U937 cells or Human Brain Micro Vascular Endothelial Cells (HBr-MIVECs). The cells were grown on glass cover slips and the attached monolayer of cells incubated first with PBS containing 0.1% BSA and 1% heat inactivated human serum or 1mg/ml Fc fragments to block Fc receptors, followed by incubation with biotinylated gC1qR and FITC-conjugated secondary reagents, as described above. After fixing for 10 min with 10 % (v/v) formalin, the cover slips were air-dried and placed face-down onto microscope slides, sealed using mounting solution (Immuno-mount), and then examined by three-dimensional imaging using deconvolution microscopy. Staining with anti-von Willebrand factor (vWF) was included to ensure the authenticity of the EC phenotype.

6.3.16 Solid-phase binding to microplate-fixed U937 cells

The ability of gC1qR and its deletion mutants to bind to U937 cells was tested using a microtitre plate-assay with fixed cells as described (Kennet et al 1980). Briefly, intact U937 cells (2×10^5 cells/well) were first attached (30 min, 22°C) onto poly-L lysine (10 µg/ml in PBS, pH 7.4) coated duplicate ELISA wells. Subsequently, the cells were fixed (30 min, 22°C) by addition of an equal volume of glutaraldehyde (0.5 % solution in PBS), and the unreacted sites quenched with glycine-BSA (100 mM Glycine, 0.1% BSA in PBS, pH 7.4). Biotinylated gC1qR or deletion mutants ranging 0-5 µg/ml were added and incubated (60 min, 22°C) and the bound gC1qR proteins detected using AP-conjugated Neutravidin, followed by pNPP substrate.

6.3.17 Effect of gC1qR on B1R and B2R surface expression

To test the effect of gC1qR on the expression of B1R or B2R, ECs were first grown overnight, fibronectin coated 6-well plates in a volume of 1 ml of EGM-2MV medium to ensure that cell attachment was complete. After blocking with Fc receptors, gC1qR or its deletion mutants were added and incubated for either 2 hr or 24 hr. The supernatant

was then removed, cells washed in fresh medium, and incubated with 5 µg/ml of either anti-B1R or anti-B2R antibody (1 h, room temp). After removing the unbound antibody, the cells were washed and further incubated (1 h, room temp) with FITC-conjugated goat anti-rabbit IgG fixed with 10% formalin and analyzed by deconvolution microscopy.

6.3.18 Statistical analysis

Student t-tests were performed using statistical software (Excel; Microsoft, Redmond, WA, USA). A value of $p=0.05$ was considered to be a significant difference. (n represents separate experiments performed in duplicates)

6.4 Results

6.4.1 The full-length gC1qR (a1-282 residues) is located on the cell surface

Previous studies have shown that the “mature form” of gC1qR (residues 74-282) was present on the cell surface. However, the presence of the full-length gC1qR (residues 1-282) was not studied on the assumption that the full-length was synthesized as a “pre-pro” protein in the mitochondria and then cleaved to generate the membrane associated “mature” protein. To determine if the full-length gC1qR was localized on the membrane, immunofluorescence studies were performed using a monospecific polyclonal IgG (pAb UN15) recognizing a synthetic peptide derived from the N-terminal residues 1-74. As shown in Figure 6.1, this antibody was able to stain the EC cell surface, and this staining was found to colocalize with that of mAb 74.5.2, which recognizes the HK site located within residues 204-218 of gC1qR

gC1qR, being a membrane associated protein, can also be secreted into the pericellular milieu by proliferating stressed cells. It has been shown previously that this soluble gC1qR is capable of binding U937 cells (Peterson et al, 1997) in a dose dependent manner. We performed a series of cell staining experiments to examine whether sgC1qR also binds to ECs. When biotinylated sgC1qR was incubated with ECs, it was able to bind to the ECs dose dependently as the concentration of gC1qR increased. Confirmation of this binding prompted us to see whether this cell attachment is mediated via residues 174-189. Hence the cells were incubated with the gC1qR deletion mutant 174-180. The results revealed that the deletion mutant 174-180 bound poorly to the ECs (figure 6.3) suggesting that it is this domain that is involved in the cell attachment. Unbiotinylated gC1qR was used as a control.

Co-localization of the “Mature” and “Full-length” gC1qR on Endothelial Cells

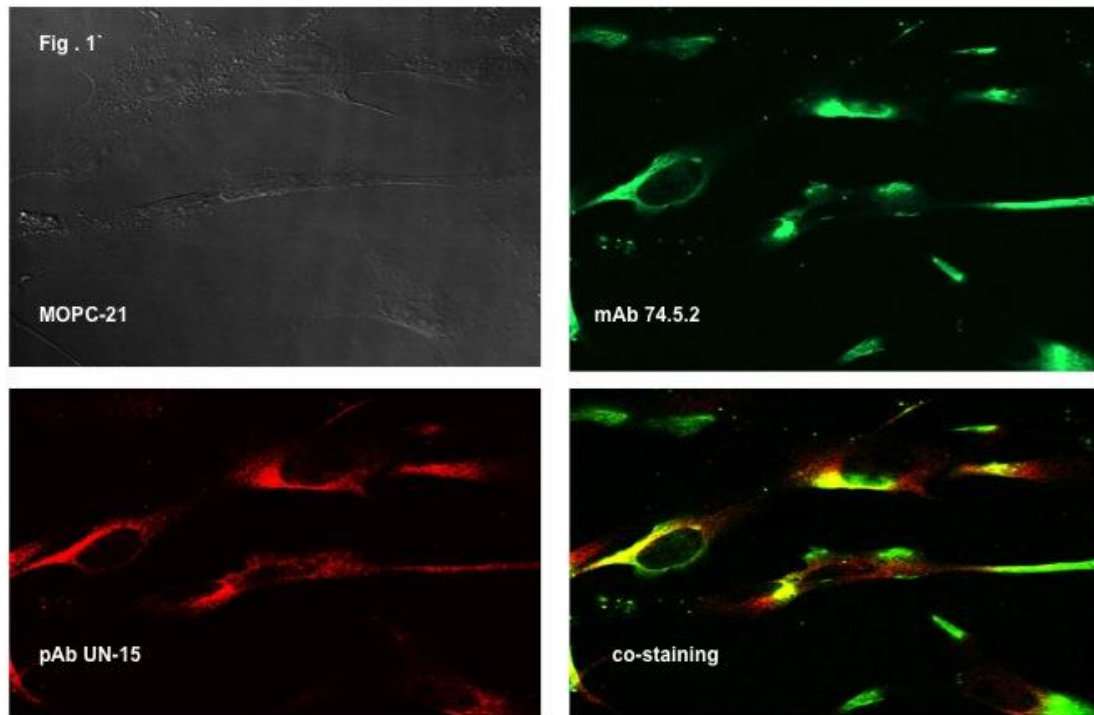


Figure 6.2: Co-localization of the mature and full-length gC1qR on the cell surface of ECs. Human brain microvascular cells were grown on glass cover slips and the attached monolayer of cells were incubated first with PBS containing 0.1% BSA and 1 mg/ml Fc fragments to block Fc receptors, followed by incubation with either Mouse IgG1, kappa monoclonal (MOPC-21), mAb 74.5.2, or pAb UN15. The bound antibodies were then probed with either Alexa-488-F(ab')₂ anti mouse (B) or Alexa 594-F(ab')₂ anti-rabbit antibodies (C). The merged picture (D) is that of the staining with mAb 74.5.2, and pAb UN15. The image is a representative of 3 such experiments. The control staining with rabbit non-immune IgG, was similar to that in (A), and is not included.

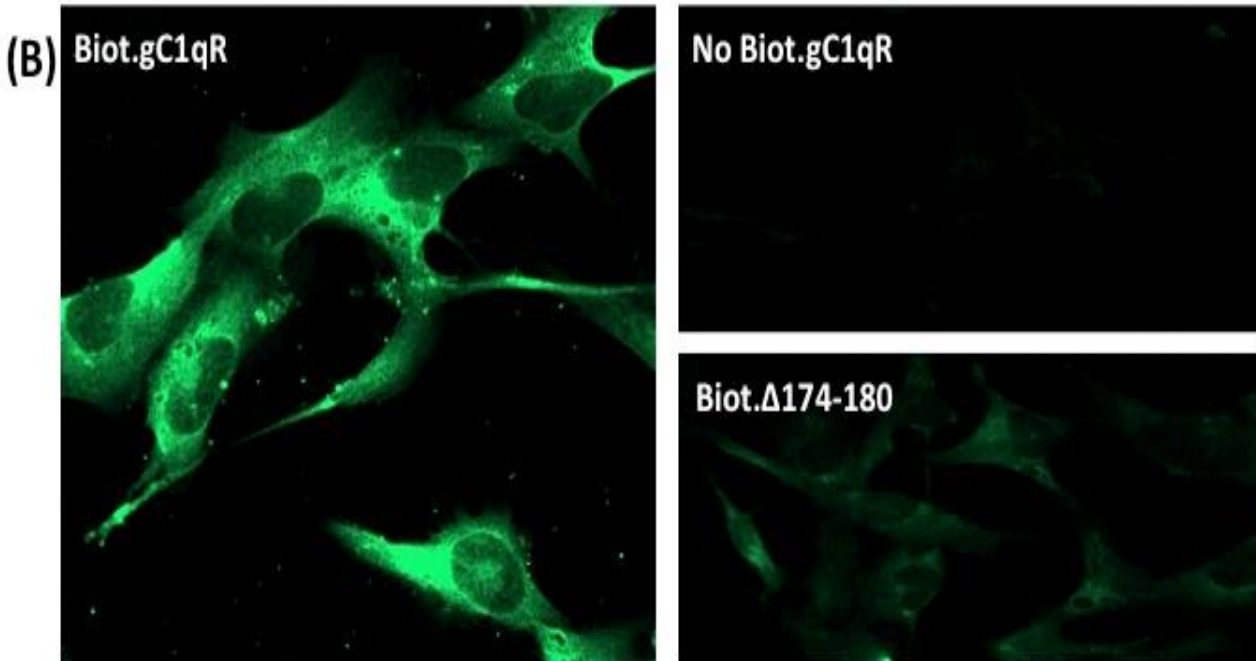
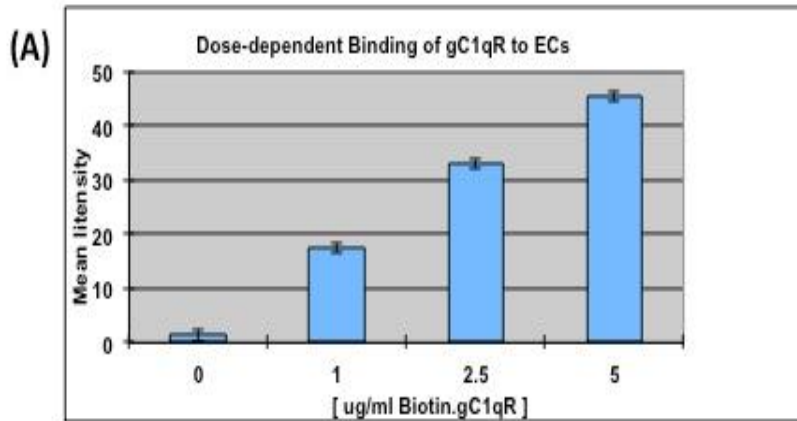


Figure 6.3. Soluble gC1qR binds to ECs. a) Cells were first stained with biotinylated gC1qR ranging from 0-5 $\mu\text{g}/\text{ml}$) and the fluorescence intensity of each staining ($n=2$) was plotted to show dose-dependence. **b)** The comparative immunofluorescence staining between biotinylated gC1qR (5 $\mu\text{g}/\text{ml}$), and biotinylated gC1qR deletion mutant lacking residues 174-180 ($\Delta 174-180$) is depicted. The negative control was that of ECs incubated with 5 $\mu\text{g}/\text{ml}$ unbiotinylated gC1qR.

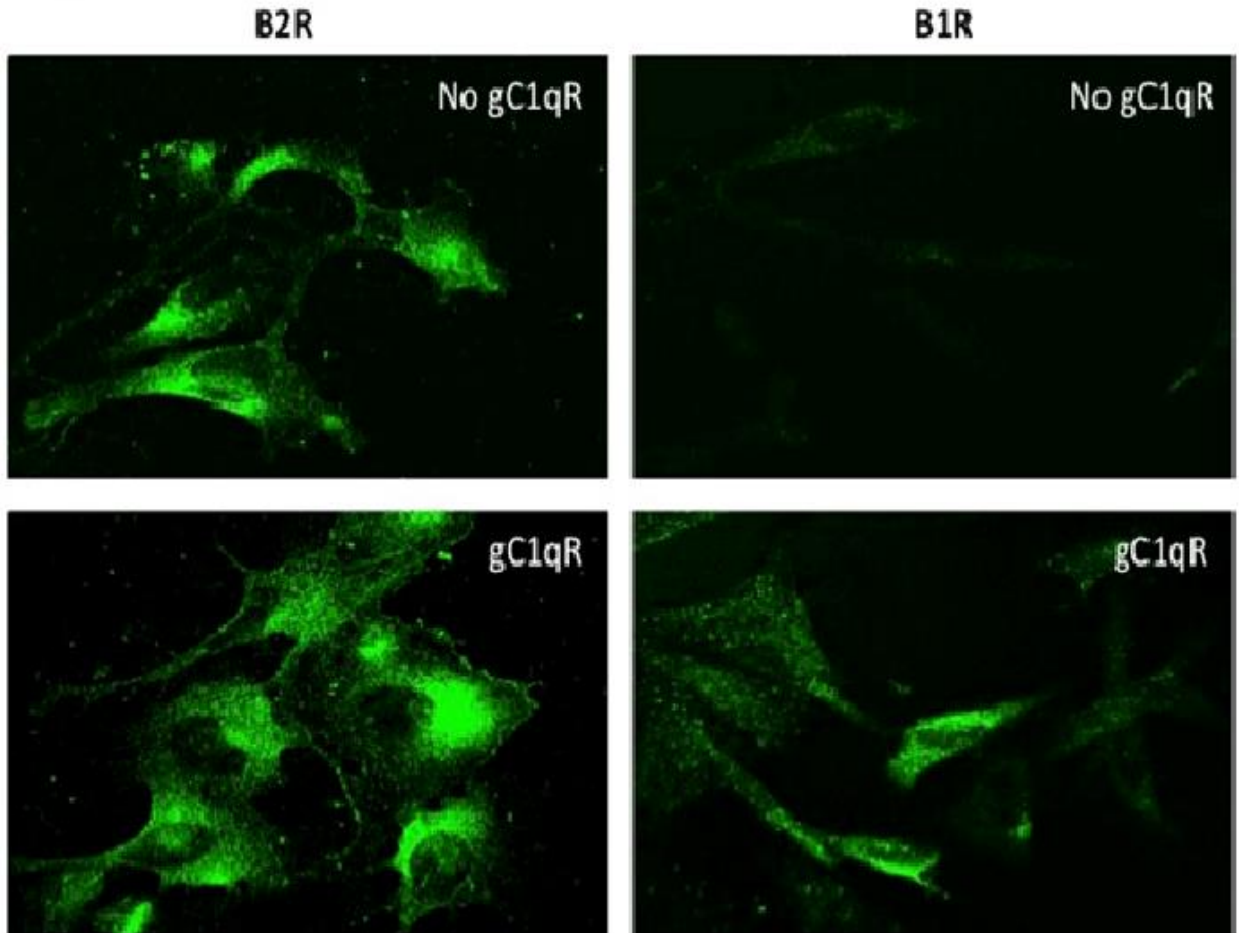


Figure 6.4: Soluble gC1qR induces the expression of B1R. Endothelial cells were first grown for 24 h as described in the legend to Figure 6.1, until full attachment and cobblestone formation was achieved. The cells were then cultured with or without either 5 μ g/ml gC1qR for an additional 24 h. The expression of either B2R or B1R was then examined by incubation of the cells with the corresponding antibodies and analyzed by deconvolution immunofluorescence microscopy

6.4.2 gC1qR binds to endothelial cells and induces the expression of B1R

Activated cells or cells undergoing proliferation are known to secrete a soluble form of gC1qR, which is structurally and functionally similar to gC1qR purified from cell membranes (Peterson et al., 1997). However, the role of the soluble gC1qR is not completely understood. We have shown previously that soluble gC1qR, purified from culture supernatants of U937 cells, can bind to intact U937 cells and this binding was similar to that obtained with recombinant gC1qR (Peterson et al., 1997). Here, we have used immunofluorescence staining and deconvolution microscopy to show that

recombinant and biotinylated gC1qR can also bind to ECs (Figure 6.3) in a manner that is specific and dose-dependent and can be blocked by 10 molar excess of its unlabeled counterpart (data not shown). In order to assess the biological implications of this binding, we first determined the optimal concentration for saturation, which was in the range of 2.5 to 5 $\mu\text{g/ml}$. Then, experiments were performed to examine what effect such binding has on B2R and/or B1R expression. While B2R is constitutively expressed, and ECs cultured alone therefore stained very strongly with anti-B2R as expected, there was nonetheless, a modest and quantifiable increase (~ 1.5 -2 fold) in B2R surface expression when the ECs were cultured for 24 h in the presence of gC1qR (Figure 6.4). In contrast, while ECs have normally small amounts of B1R on their surface and is reflected by a very weak staining, the staining with anti-B1R increased considerably by increasing concentrations of gC1qR, reaching maximal staining at 5 $\mu\text{g/ml}$ gC1qR (Figure 6.4).

gC1qR was able to bind to the ECs in a dose-dependent manner. ECs cultured with gC1qR led to the induction of B1R. This prompted us to determine the sites/domain on gC1qR that are involved in binding to ECs and inducing B1R expression. Biotinylated gC1qR deletion mutants were incubated with ECs and probed with AP-streptavidin. All the deletion mutants were able to bind at different degrees to fixed ECs. However, $\Delta 174$ -180, $\Delta 154$ -162 $\Delta 144$ -162 and $\Delta 196$ -202 bound poorly compared to WT gC1qR, suggesting that these domains are involved in binding to ECs and triggering B1R expression. Figure 6.5 displays the deletion mutants mentioned above bound to ECs with the same intensity as no gC1qR implying that they these residues are key players in B1R expression. The microscopy results revealed that deletion mutant $\Delta 144$ -148 and $\Delta 212$ -223 bound to ECs the strongest out of the variants. However, none of the domains appeared to engage with the ECs as well as WT gC1qR, suggesting that the overall structure of gC1qR contributes to B1R expression.

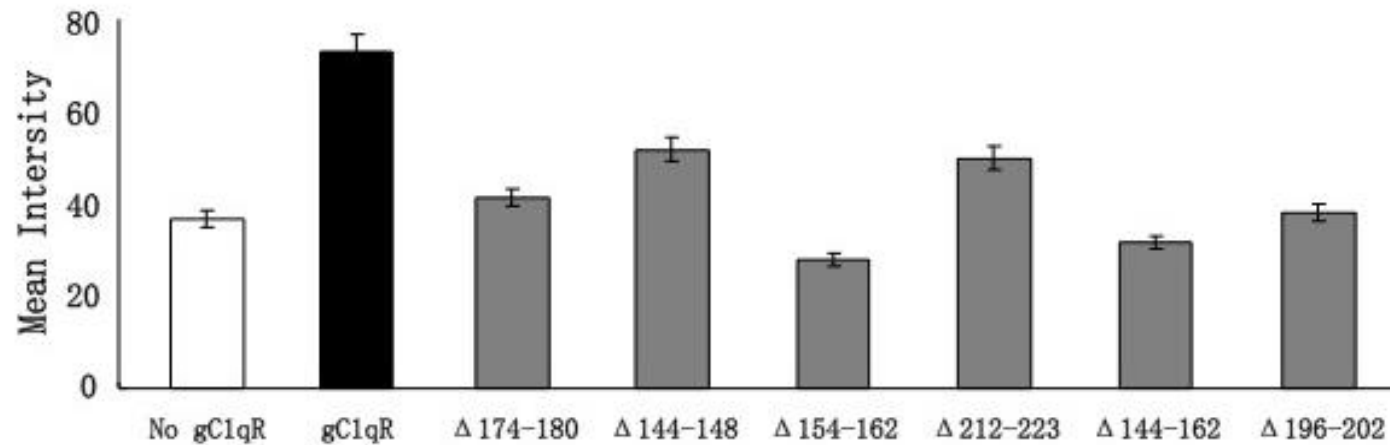
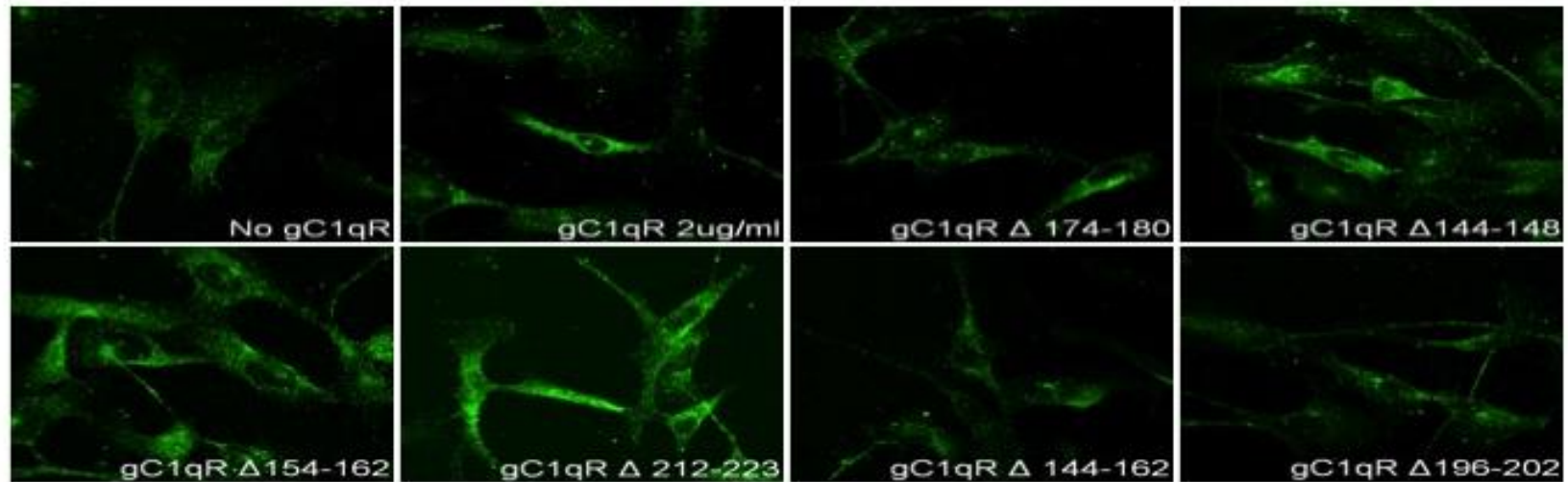


Figure 6.5: Comparison of the effect of WT gC1qR and its deletion mutants on B1R expression. The binding of biotinylated (2 μ g/ml) gC1qR and various deletion mutants (Δ) to ECs was assessed by immunofluorescence microscopy (top figure). The mean intensity of each staining is plotted (bottom figure) for comparison. The experiment is a representative of 3 experiments run in triplicates.

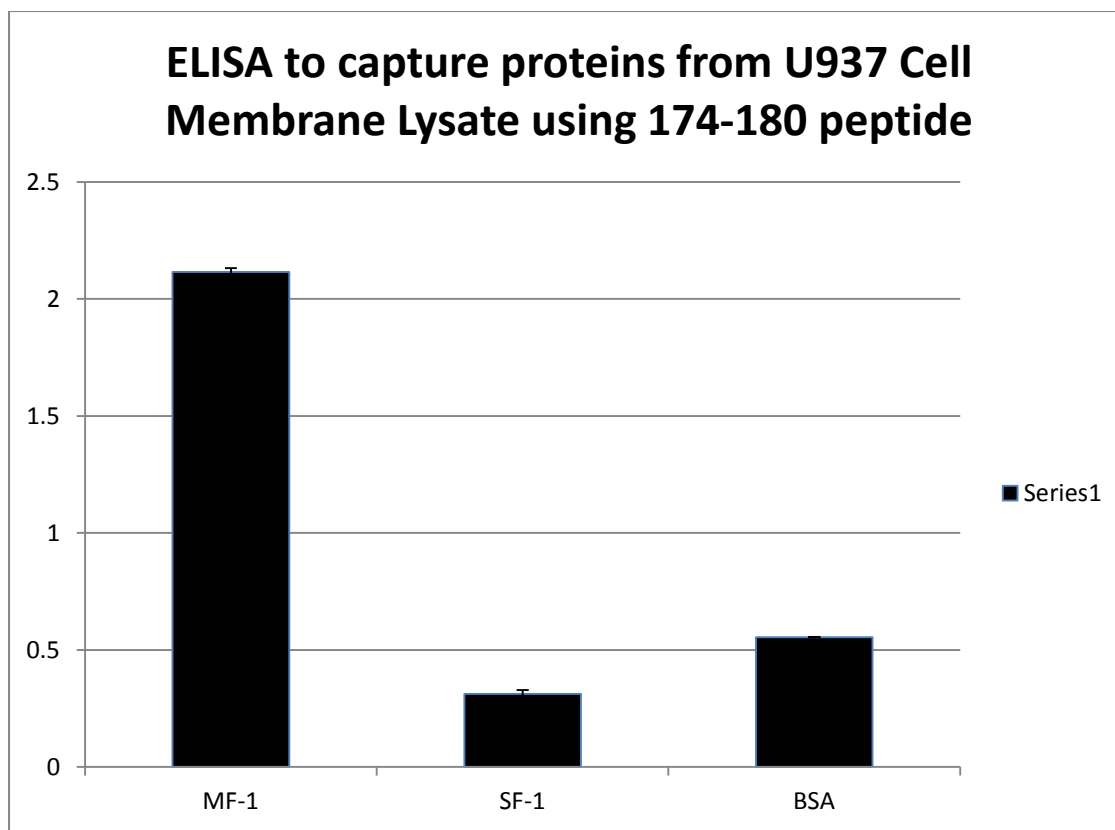


Figure 6.6: Protein(s) captured from U937 cell lysate using 174-180 peptide. ELISA-based antigen capture assay in which duplicate wells of a microtiter plate were first coated with 100 μ l (100 μ g/ml) of either gC1qR peptide MF-1 174-180 or peptide SF-1 144-162. After blocking with 1% BSA the wells were incubated (overnight, 4^o C) with 100 μ l of surface biotinylated U937 membrane proteins and the captured protein(s) subsequently detected with alkaline phosphatase-conjugated Neutravidin and developed following standard ELISA procedures.

6.4.3 ELISA to capture proteins from Biotinylated U937 membrane cell lysate

MF-1 peptide corresponding to the sequences 174–180 of gC1qR was coated on microtitre plates. When biotinylated U937 membrane lysates were added to the wells and probed with AP-Streptavidin. MF1 bound greatly compared to its control sample SF-1 (a peptide sequence extracted from the solution face of gC1qR). BSA was used as a negative control. As shown in figure 6.6 MF-1 bound protein(s) from the U937 lysate at a remarkable rate of nearly 6 folds greater than the control peptide indicating MF-1 was capturing a protein from the lysate.

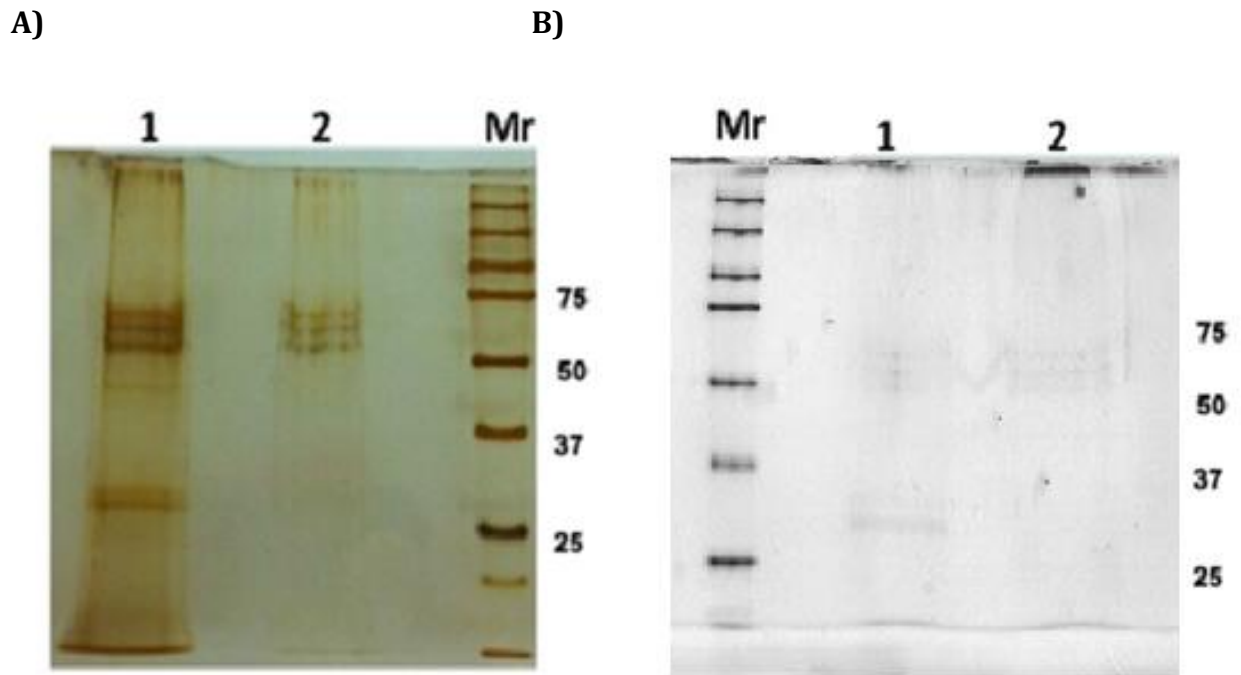


Figure 6.7: Intact gC1qR and gC1qR peptide 174-180 recognize the same membrane protein. **a)** Proteins expressed from Cells grown in Serum, **b)** Proteins expressed from Cells grown in Serum free media. (Capture assay run on a 10% SDS gel under reducing conditions.) Each lane represents 41,000,000 cells) 125×10^8 were washed and resuspended in 3ml solubilisation buffer. 100ug/well of 174-180 peptide was coated on 10 wells, and 5ug/ml of gC1qR on another 10 wells and left at 37 degrees for 1.5 hours. Contents were discarded and wells were blocked with 3% BSA for 1 hour. Wells were washed 3 x in TBS-T and 100ul of cells were added to each well and incubated overnight at +4. After the contents were discarded and wells were washed 3x in TBS-T, 100ul of glycine was added to each well and left on a shaker for 1 hour at room temperature. The glycine was pooled to give a total volume of 1ml for each protein sample, and this was added to 4ml cold Acetone, (4x Acetone to protein sample required). The mixture was left at -20 for 1 hour to allow precipitation of the proteins. Next, the samples were spun at 13,000 RPM for 10 minutes. Sample buffer was added to the pellet and run on a SDS-GEL. **A)** Lane 1: 174-180 peptide capturing protein from U937 cells grown in Serum. Lane 2: gC1qR used as a control to capture protein from U937 cells grown in Serum. **B)** Lane 1: 174-180 peptide capturing protein from U937 cells grown in Serum free media. Lane 2: gC1qR used as a control to capture protein from U937 cells grown in Serum free media

6.4.4 U937 Cell line used to identify proteins binding to 174-180 sequence of gC1qR

The U937 cell line is derived from a histiocytic lymphoma male patient and displays many monocytic characteristics. It can be used as a model to study the differentiation of monocytes to macrophages. The cell line produces a range of cytokines and growth factors including IL-1 β , a pro-inflammatory cytokine, which is involved in inflammation and works to recruit leucocytes to fight infection. GM-CSF (Granulocyte Macrophage Colony Stimulating Factor) is a growth factor expressed by U937 cells. It stimulates the proliferation and differentiation of precursors of monocytes. GM-CSF has also shown to stimulate the production of the anti-inflammatory cytokine IL-10 in U937 cells. Another protein expressed by U937 cells includes VPF (Vascular Permeability Factor), which is a protein secreted by tumor cells that provides a blood supply that supports the metastasis of tumors. MDSF (Monocyte derived scattering factor) is also released by U937 cells which increases the motility of the cell

6.4.5 Proteins captured from U937 Cell lysate using 174-180 peptide

Once the ELISA performed using the 174 – 180 peptide to capture from the U937 lysate showed indication of a pull down (Figure 6.6), the next step was to identify what molecule(s) this conserved sequence was capturing. Using glycine to break the bonds between the peptide and the captured protein(s), the sample of interest was eluted into acetone to allow precipitation of the protein(s). Once run on a 12% SDS gel and visualised using Silver Stain gel, 3 bands were observed corresponding between the 50 and 75kDa marker. A doublet was also seen which appeared to be above the 25kDa marker. Figure 6.7a shows the capture assay performed on U937 cells cultured in serum media. After confirmation of the 3 bands, it was proposed to ensure that any proteins being captured were not picked up from the serum. Such a possibility could occur due to serum proteins binding to proteins expressed by U937 cells, therefore enabling them to remain in the membrane lysate. To eliminate this possibility, the cells were grown in serum free media. As figure 6.7b 18 shows, the same 3 bands were expressed, which confirmed that the captured proteins were not serum-derived. The bands in figure 6.7, lane 1 (174 – 180) were much more abundant than the protein bands captured by gC1qR in lane 2, this was probably due to other proteins in the lysate

binding to gC1qR which could have masked the 174 – 180 binding site, therefore resulting in a lower amount of protein being pulled down. The gel was subjected to mass spectromic analyses. However, due to high keratin contamination, the bands were not able to be detected. The next mode of action was to have the pellet itself digested which would greatly reduce keratin contamination. However, this produced many protein hits (see appendix) which could have again been masking the protein(s) of interest, indicating that the sample was not pure enough. Both gels were run under reducing conditions, therefore it was interesting to see how the protein(s) behaved under non reducing conditions on a 6% gel. However after such a experiment was performed, no bands were seen on the gel (data not shown) which suggested the point that the protein exists as a trimer and that under non reducing conditions, is too large to enter the gel.

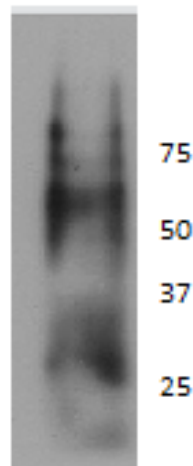


Figure 6.8: Western Blotting to show 174-180 capturing U937 cell surface proteins grown in Serum free media. U937 cells were washed and resuspended in 5ml DPBS, to which 100ul of DMSO-LC Biotin Mixture (3mg/ml) was added and left on a shaker for 1-2hrs at +4. Cells were solubilised. Protein capture assay and protein precipitation was carried out as described previously and samples were run on a 10% SDS gel. The captured proteins were transferred onto a PVDF membrane and probed with HRP-Streptavidin.

6.4.6 Western Blotting to visualise 174- 180 peptide capturing proteins from biotinylated U937 membrane lysate

Once the Silver Staining of the precipitated captured protein(s) confirmed the presence of a trimer between 50 -75kDa and a doublet above the 25kDa marker, we wanted to confirm that these identified proteins were essentially expressed on the surface of U937 cells. As the cell lysate was biotinylated, western blotting and probing with a streptavidin conjugate would verify the surface location of these proteins. As shown in figure 6.8, the western blotting detected the same trimeric bands between the 50 and 75kDa marker, as well as the doublet corresponding above the 25kDa marker. This validated that the proteins being captured from the lysate were on the surface of these U937 cells.

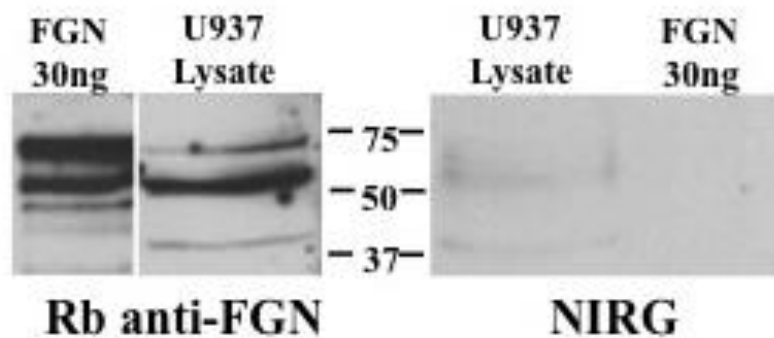


Figure 6.9: Western blotting to detect fibrinogen: Protein capture assay and protein precipitation was carried out as described previously of the U937 cell lysate and samples were run in duplicates run on a 10% SDS gel with the addition of Fibrinogen (FGN) in duplicates too . The gel was transferred onto PDVF membrane as described and blocked in 5% non fat milk in TBS for 1 hour at room temperature. The blot was cut to separate the transferred bands and one strip was probed with rabbit anti-Fibrinogen and the other strip with Non Immune Rabbit IgG. Following three washes with TBS-T for 10 minutes each, the secondary antibody used was rabbit anti goat. The bound antibodies in turn were visualized by chemiluminescence horseradish peroxidase-conjugated species-specific antibody followed by reaction with 4-chloro-1-naphthol substrate The three bands between 75 and 50 kDa in lanes 1 and 2 in (B) are the α , β , and γ chains of fibrinogen, and are recognized by anti-FGN IgG and not by non-immune IgG (NIRG).

The protein capture assay analysed by SDS-PAGE and Western blotting revealed three bands corresponding between 50-75kDa. Such a three-chain molecule and its molecular weight suggested that this could potentially be fibrinogen. gC1qR is known to bind to fibrinogen (Lu et al, 1999) specific to the D domain and the γ chain also contributing to the interaction. The fact that this gC1qR/FGN interaction was possibly occurring on the cell surface, encouraged us to identify this three chain protein. We performed a western blot in duplicates, identical to the one mentioned with the addition of FGN as a positive control. The one transferred lysate was probed with anti-FGN IgG and the other one with Non Immune Rabbit IgG (NIRG) as a negative control and. As shown in figure 6.9, anti-FGN Ab was able to pick FGN as well as all three chains, α , β and γ of FGN from the U937 membrane cell lysate. NIRG, as expected, was not able to pick up either of the transferred bands confirming that three bands observed in figure 6.9 are the α , β and γ chains of FGN.

6.4.7 Anti-fibrinogen IgG inhibits gC1qR binding to U937 cells

To further validate the gC1qR binding to cell surface FGN, we performed a standard ELISA using microtitre fixed U937 cells to see if anti-FGN was able to inhibit the binding of gC1qR to FGN. gC1qR binding to FGN was reduced in the presence of anti-FGN Ab when compared to NIRG and PBS, which were used as positive controls further validating the identification of this cell surface protein to be FGN.

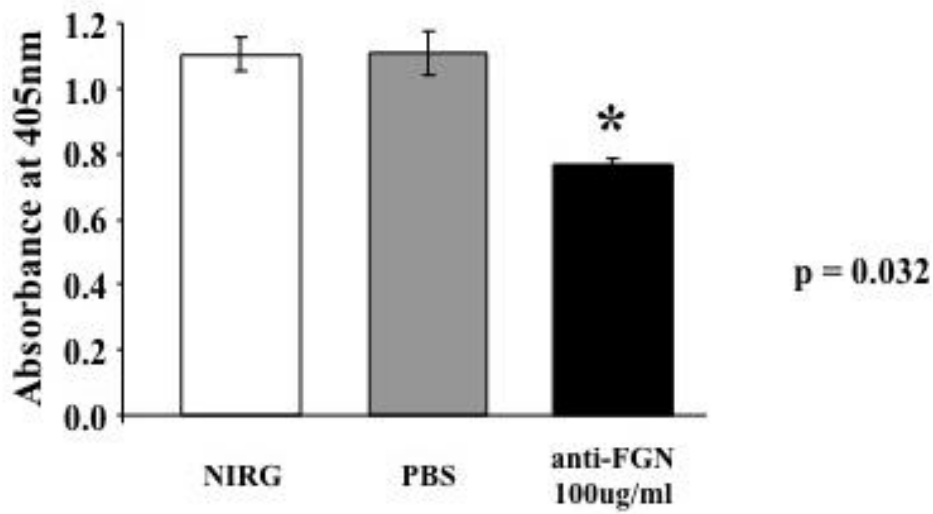


Figure 6.10: Anti-fibrinogen IgG inhibits gC1qR binding to U937 cells. The binding of biotinylated gC1qR to microtiter plate-fixed U937 cells was assessed by standard ELISA to examine the ability of gC1qR to bind U937 cells in the presence or absence of anti-fibrinogen IgG or non-immune rabbit IgG as control. U937 cells were first attached to 96-well microtiter plates using poly-L-Lysine and fixed as described (Kennet, 1984; Ghebrehiwet et al., 1996). Anti-FGN IgG in PBS, NIRG and PBS were added to the wells and incubated at 37°C for a hour. The wells were washed three times in TBS-T and 3µg of biotinylated gC1qR was added to each condition and incubated for a further hour for 37°C. Bound protein was detected using AP-Streptavidin.

Isotype Control

FGN-gC1qR Colocalization

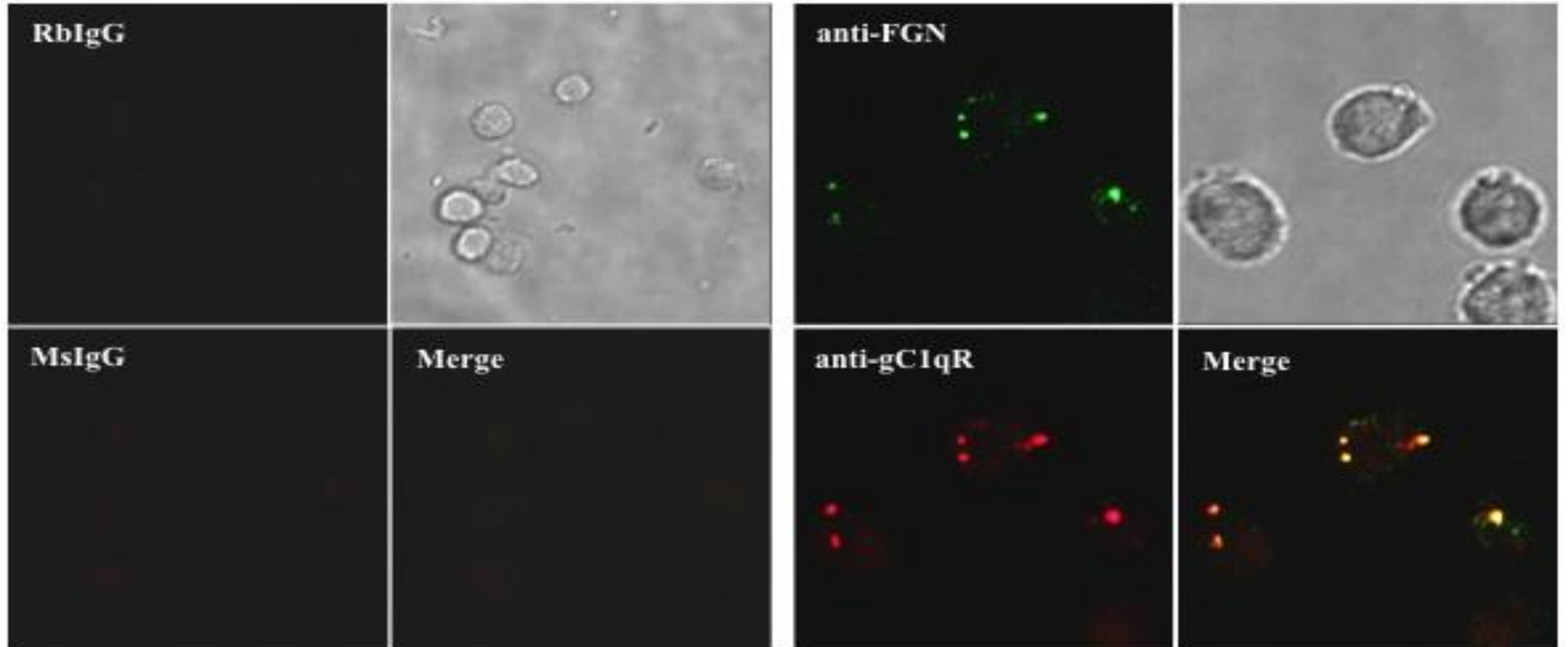


Figure 6.11. Fibrinogen and gC1qR are co-localized on the surface of U937 cells. U937 cells that were grown to confluence in serum-free medium, were first incubated with either 5 $\mu\text{g}/\text{ml}$ rabbit non-immune IgG (RbIgG) or MOPC-21 (MsIgG) or rabbit anti-fibrinogen or mAb74.5.2 anti-gC1qR. The bound antibody was then visualized using Alexa-488-(green) or Alexa 594 (red)-conjugated F(ab')₂ secondary antibody. After fixing, the cells were analyzed by immunofluorescence staining and deconvolution microscope.

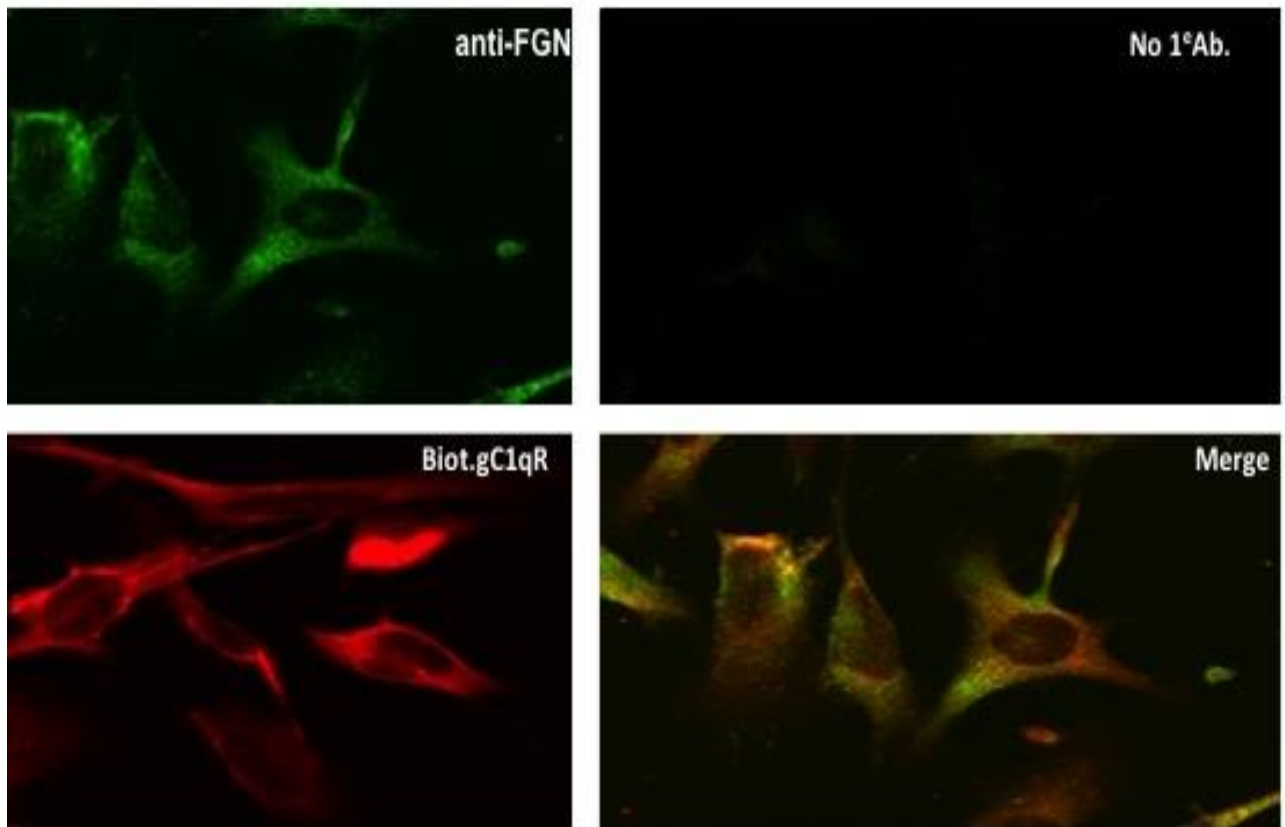


Figure 6.12. Co-localization of gC1qR and fibrinogen on the endothelial cell surface. (A) ECs were grown on cover slips and incubated with either ant-FGN (anti-FGN), biotinylated-gC1qR (Biot.gC1qR) or both (merge). Cells incubated without primary antibody (No 1°Ab) were used as negative control.

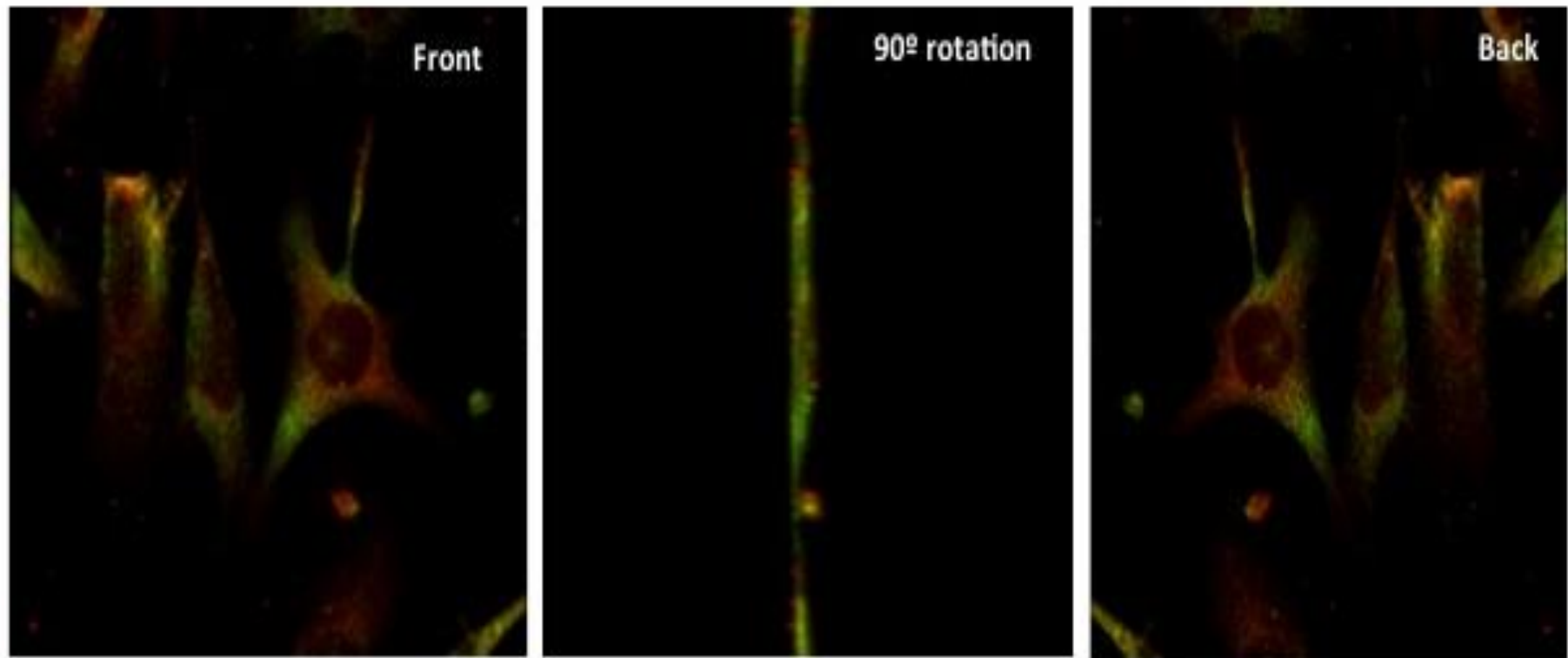


Figure 6.13: Co-localisation of FGN and gC1qR: Cells showing co-localization of FGN and gC1qR (merge) were subjected to 3D rotation to show staining of cells as seen from the front (or top), the side (90° rotation), or back (360° rotation) views.

6.4.8 Co-localisation of gC1qR and FGN on the cell surface of U937 cells and ECs

We sought to visualize the colocalisation of FGN and gC1qR on the cell surface of U937 cells and ECs. We first verified the presence of FGN and gC1qR individually by incubating the cells with anti-fibrinogen and biotinylated gC1qR followed by anti-gC1qR 74.6.2. Probing of the antibodies using Alexa-488-(green) or Alexa 594 (red)-conjugated F(ab')₂ secondary antibody and visualizing using deconvolution microscopy showed the clear presence of both FGN and gC1qR on the cell surface of U937 cells and ECs (figure 6.11 and 6.12). To verify the interaction of these two proteins on the cell surface, we also investigated the colocalisation of FGN and gC1qR using immunofluorescent microscopy. The analysis of this colocalisation was confirmed by the merging of anti-FGN and gC1qR (figure 6.11 and 6.12) clearly showing the interaction between gC1qR and FGN. The staining on both types of cells appeared to be robust implying that cell surface FGN is the ligand for soluble gC1qR. The negative control used in both experiments was NIRG which showed no binding (figure 6.11 and 6.12). The staining of FGN and gC1qR together was also subjected to rotation to visualize the staining from the top, side and back view (figure 6.13).

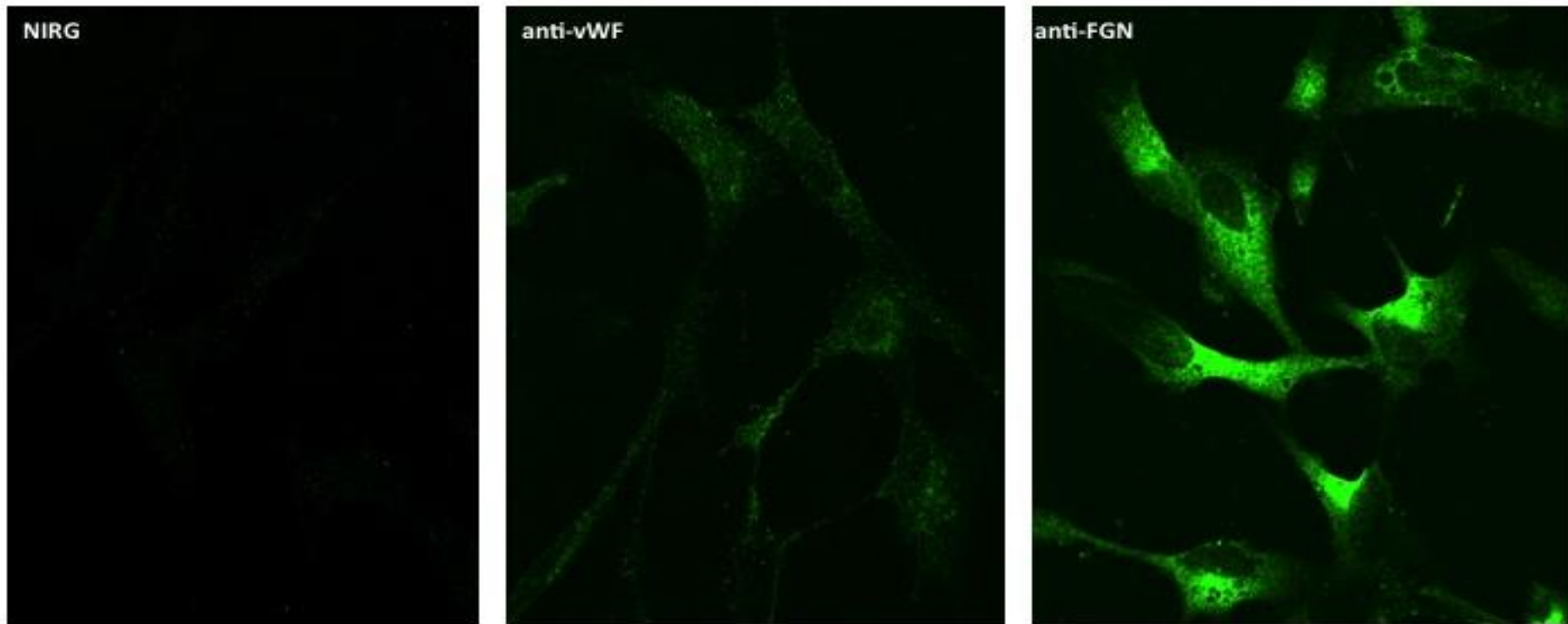


Figure 6.14: Comparison of the expression of fibrinogen and von Willebrand factor on EC surface. Cells were grown on cover slips and incubated with either 5 $\mu\text{g}/\text{ml}$ anti-fibrinogen (C), anti-von Willebrand factor (B) or species-matched IgG (A). The bound antibody was visualized using Alexa-488-F(ab')₂ secondary antibody as described in Methods.

6.4.9 Domain 174-180 is the primary attachment site for soluble gC1qR

Soluble gC1qR is able to bind to various cell types including U937 (Peterson et al., 1997) and ECs. However, neither the domain of gC1qR that allows for such binding nor the cellular entity that is the site for gC1qR binding is known. In an effort to identify the gC1qR domain that is the potential binding site, we compared the binding of wild type gC1qR with 10 gC1qR deletion (Δ) mutants lacking highly charged surface exposed domains. As shown in Figure 6.5, gC1qR lacking residues 174-180 or 144-162 and 154-162 consistently showed diminished binding to ECs suggesting that at least two sites exist: one at 174-180 and another in an overlapping site between 144-162 and 154-162. Because subsequent binding studies using microtitre plate fixed U937 cells showed consistently a diminished binding with gC1qR Δ 174-180, we generated two synthetic peptides: one corresponding to residues 174-180 and another to 144-162. These peptides were then used in an antigen-capture ELISA to pull down solubilized membrane protein(s) from surface biotinylated U937 cells. As shown in Figure 6.6, only peptide 174-180 was able to pull down a biotinylated membrane protein(s). To identify of the captured protein, pull down experiments were performed using intact gC1qR and peptide 174-180. When the captured U937 membrane proteins were analyzed by Western blotting under denaturing and reducing conditions (Figure 6.9), a molecule with a chain structure and molecular weight that resembled fibrinogen (FGN) was visualized.

6.4.10 Cell surface expressed fibrinogen is the ligand for soluble gC1qR

The presence of FGN on cell surfaces has not, to our knowledge, been reported before. Therefore to examine the presence of FGN on the cell surface, we used anti-FGN to examine the presence of FGN on U937 using microplate-fixed U937 cells grown either in complete medium containing fetal bovine serum or serum-free growth medium. Results showed a strong anti-FGN binding when compared to isotype- and species-matched IgG (data not shown). Then experiments were done to determine if anti-FGN antibodies could inhibit gC1qR binding to microplate-fixed U937 cells. As shown in Figure 6.10, a statistically significant inhibition ($p=0.032$) was observed when cells were pretreated with anti-FGN prior to incubation with gC1qR. Subsequent

immunofluorescence experiments confirmed the presence of FGN on U937 cells (Figure 6.11) and ECs (Figure 6.12 and 6.13).

6.5 Discussion

gC1qR is synthesized as a full-length protein of 282 residues, which is then cleaved at position 73-74 to generate the membrane-bound mature form (residues 74-282) (Ghebrehiwet et al., 1994). However, when a monospecific polyclonal antibody recognizing a peptide (residues 50-63) in the gC1qR region covered by residues 1-73 was used in immunofluorescence studies, a robust membrane staining was observed (Figure 6.1) indicating the presence of either the full-length protein or a fragment that contains residues 1-73. This staining was comparable to the staining observed with mAb 74.5.2, which recognizes residues 204-218. Thus, in addition to its localization inside the cell, the full-length molecule is localized on the membrane (Kim et al., 2009) probably via a CRAC (cholesterol recognition amino acid consensus) motif present on gC1qR.

Most proliferating or activated cells are known to overexpress and secrete gC1qR that can potentially modulate a diverse range of biological functions including angiogenesis (Bossi et al., 2011), vascular permeability (Joseph and Kaplan, 2005) (Ghebrehiwet et al., 2013) and inflammation (Ghebrehiwet et al. 2013) through complement activation and the kinin/kallikrein systems (KKS). This is partly due to the susceptibility of gC1qR to proteolytic cleavage by enzymes in plasma, or membrane-associated enzymes such as Membrane type 1 metalloprotease (MT1-MMP) as shown earlier (Rozanov et al., 2002). Soluble gC1qR (either recombinant or purified from cell cultures), can bind to U937 cells (Leeb-Lundberg et al., 2001). Here, we wish to (a) examine if gC1qR also binds to ECs; (b) identify the surface molecule(s), which serves as the gC1qR site; and (c) assess whether gC1qR can serve as an autocrine/paracrine signal for the induction of B1R, which, unlike B2R, is induced by IL-1 β (Menke et al., 1994; Hess et al., 1992; Mceachern et al., 1991; Marceau and Regoli, 2004).

Here, we show that gC1qR binds to ECs in a dose-dependent manner (Figure 6.2 and 6.3), enhances B2R expression, and induces B1R expression on ECs (Figure 6.4). When binding of wild type gC1qR to U937 cells or ECs was compared with a range of gC1qR deletion mutants each lacking in a highly charged region exposed on the surface of the molecule (Jiang et al., 1999; Ghebrehiwet et al. 2002), the binding of gC1qR deletion mutants lacking in residues 174-180, 144-162 and 154-162 were significantly reduced

when compared to wild type gC1qR (Figure 6.5). However, only peptide 174-180, and not 144-162, was able to recognize a membrane protein from U937 cells. Thus, initial gC1qR interaction site may be located on the domain 174-180 with subsequent multiple interactions via other sites including residues 144-62. While both gC1qR- and peptide 174-180 captured a membrane protein from U937 cells, peptide 144-162 did not. The captured protein(s), in turn, appears to be surface bound fibrinogen. In the pull-down experiments (Figure 6.7), a three-chain molecule similar to fibrinogen appeared on SDS-PAGE. In addition, anti-fibrinogen antibodies inhibited binding of gC1qR to U937 cells. Both U937 (Figure 6.11) and ECs (Figure 6.12 and 6.13) stained strongly for fibrinogen (Figure 6.14). Recombinant gC1qR has previously been shown to bind human fibrinogen and interfere with its polymerization (Lu et al., 1999). The binding site for gC1qR was found to be on the D domain of fibrinogen/fibrin, with the carboxy-terminal segment of fibrinogen/fibrin γ chain being important for this interaction, suggesting a potential role for gC1qR in modulating fibrin formation at the site of injury or inflammation (Lu et al., 1999). Therefore, the gC1qR/fibrinogen interaction taking place on the EC surface represents a novel biological function for soluble gC1qR in regulating local inflammation.

Thus, soluble gC1qR can bind ECs (Guo et al., 1999). Highly activated cells such as tumor cells or cells at inflammatory sites overexpress gC1qR on their surface, as well as release a soluble form of the molecule into the pericellular milieu. Each form of the gC1qR molecule (surface expressed or soluble) has been shown to play a role in the induction of inflammation by activation of the KKS as well as classical pathway of the complement system (Peterson et al., 1997). Thus, activation of the KKS leads to the generation of BK (residues Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), which acts on the B2R to trigger vascular permeability. In humans, the half-life of BK is extremely short. It is rapidly degraded by three kininases: angiotensin-converting enzyme, aminopeptidase P, and the carboxypeptidases N (CP-N), or M, (membrane bound enzyme), which cleave the 7-8, 1-2, and 8-9 positions, respectively (Dendorfer et al., 2001, Kuoppala et al., 2000). The first degradation product of BK-des-Arg⁹-BK is more stable and possesses a high affinity for B1R. Although a detectable amount of B1R exists on the cell surface, B1R is induced by IL-1 β produced by monocytes recruited to the inflammatory site. Soluble gC1qR is capable of inducing B1R expression, and thus enhances BK-mediated

vascular permeability. It is also able to recruit C1q, a powerful chemoattractant (Leigh et al, 1998), which like activated HK (HKa), is also able to generate proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, and the chemokines IL-8 and MCP-1 (Khan et al., 2006; Van den Berg et al., 1998). Thus, at sites of inflammation or under pathological conditions such as angioedema, in which vascular permeability plays a central role, gC1qR can facilitate various pro-inflammatory cascades, and therefore, represents a suitable therapeutic target to prevent local or systemic inflammation.

CHAPTER 7:

Conclusions and future perspectives

7.1 Conclusions and future perspectives

Since its discovery from Raji cells in 1994, gC1qR has undergone numerous nomenclatures including C1q binding protein (C1BP), mitochondrial protein p33, etc. starting as a C1q binder via the gC1q domain, gC1qR has been now established as a multi-ligand binder and multi-functional protein. This aspect has been dealt with in great details in the Introduction of this thesis. There are continued debates about the localisation of gC1qR-mitochondrial, intracellular, cell surface, and extracellular? Like many proteins, the cellular localisation of these candidate receptor molecules is a subjective matter dependent on the cell type, local environment, techniques used, and the context of health and disease. The gC1qR counterpart, CRT, also went through this debate from being endoplasmic reticulum resident protein to trapped in lipid raft, and then from being on the cell surface (anchored to CD91) to being secreted. However, the fact that gC1qR is exclusive in binding to the globular head domain of C1q remains interesting and intriguing. With the availability of the recombinant forms of individual globular head modules (ghA, ghB and ghC) and a number of single residue substitution mutations, it became possible to re-examine the nature of interaction between gC1qR and gC1q domain of C1q. In addition to establishing ionic interaction between gC1q and gC1qR, the current study highlighted a central role for A chain and residue Arg¹⁶² in the protein-protein interaction. The biphasic upregulation of expression of gC1qR and C1q in adherent monocytes appears to suggest their autocrine/paracrine relationships. The downregulation of gC1qR in response to LPS challenge appears to suggest that gC1qR involvement in processes involving direct interaction with LPS is not required although C1q remains a crucial innate immune component. Experiments have been set up and are being carried out currently in our laboratory to examine if individual globular head modules, on their own, exert anti-lymphoproliferative effect or have additive effects of C1q-mediated effect on PHA-stimulated PBMCs. We are also asking the question if this effect of globular head modules or C1q can be modulated by soluble form of recombinant gC1qR. These studies are being carried out to establish a clear biological link between C1q functions and gC1qR in the regulation of inflammation and immune activation.

The novel finding of a tripartite interaction between C1q, gC1qR and DC-SIGN needed further exploration. Having established the nature of interaction between these three

crucial molecules that are associated with the HIV-1 infection of dendritic cells, experiments are in progress to examine how DC-SIGN mediated HIV-1 infection is modulated by recombinant globular head modules independently as well as in concert with gC1qR, given that both gC1qR and C1q are gp41 binders. We are culturing HEK293 and U937 cells transfected with lentivirus construct that expresses full-length DC-SIGN on the cell surface. The ability of C1q, recombinant ghA, ghB and ghC modules, and gC1qR to enhance or inhibit viral entry via DC-SIGN and viral replication in vitro is being examined. HIV-1 induced cytokine storm in the transfected U937 cell line in these assays will be monitored via qPCR analysis and cytokine array. This will be followed up by examining phosphorylation of key kinases p38, Erk1/2 and AKT, which contribute to HIV-1 induced immune activation using commercially available antibodies.

An indicator anti-HIV-1 assay is also being carried out that relies on the generation of blue foci of infection in the cellular background. HIV-1 infection is quantified using TZM-bl cells, which express luciferase as well as β -galactosidase genes under the control of the HIV-1 LTR promoter. TZM-bl cells (6×10^3) are grown in a 96-well tissue culture plate for 24 h. In separate tubes, 100 TCID₅₀ units of HIV-1 (strains: HIV-1LAI, HIV-1 IN93/905, HIV-1 98/IN/017 and HIV-1 96USNG31) are pretreated with indicated concentrations of C1q, globular head modules and gC1qR and the opsonised virus is allowed to infect TZM-bl cells. After 4 h, excess virus is washed with 50mM PBS, fresh medium was added, and then cells are incubated for an additional 48 h. Next, cells are washed twice with PBS, fixed in 1% glutaraldehyde for 10 min at room temperature followed by staining with X-gal staining solution (10ml PBS with 1mg/ml X-gal dissolved in DMF, 100mM potassium ferricyanide, 100mM potassium ferrocyanide and 1mM MgCl₂) for 24 h at 37°C. The blue stained (β -gal expressing) foci are counted under the microscope.

Quantitation of p24 levels as a marker of viral replication is being carried out in culture supernatants. Different concentrations of soluble recombinant proteins will be pre-incubated with 100 TCID₅₀ of HIV-1 96USNG31 for 1h before addition to HEK-DC-SIGN cells. After 4h, residual virus is removed, cells are washed and fresh medium added. On day 3, 6, 9 and 12, 100 μ l supernatant was collected. Viral replication will be determined by the HIV-1 p24 Antigen Capture Assay ELISA (XpressBio). The cytokine and growth

factor analysis will be done using Cytokine Array 1 kit for IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IFN- γ , TNF- α , MCP-1, VEGF, and EGF. These experiments will help us understand the roles played by C1q and gC1qR in association with DC-SIGN in HIV-1 infection of dendritic cells and perhaps by-stander cells. Having established gC1qR as a pattern recognition receptor of viral proteins using HCV core protein and HIV-1 gp41 as a model system, the identification of complementary binding sites may have implications in therapeutic strategies. Again, after optimising the infection assays, these therapeutic peptides can be used in the HIV-1 infection assay, as described above.

The identification of a role for gC1qR as an inducer of B1R on the endothelial cells is very interesting. Since tumour cells overexpress gC1qR which are secreted as well as surface-bound, they can give rise to basal level of inflammation that can promote tumourogenesis and metastasis involving activation of KKS as well as the classical pathway. Bradykinin mediated initiation of vascular permeability via B2R, and subsequently via B1R offers an excellent target for therapeutically controlling these inflammatory processes. Thus, IL-1 β mediated upregulation of B1R surface expression is acted upon in concert by gC1qR. This paves the way to identify agonists and antagonists. It will be worth examining if adherent monocytes overexpress gC1qR and B1R in tandem and if they are altered by pro-inflammatory stimuli such as LPS and IL-1 β .

8. References

- ABBAS, A. K., LOHR, J., KNOECHEL, B. & NAGABHUSHANAM, V. 2004. T cell tolerance and autoimmunity. *Autoimmunity Reviews*, 3, 471-475.
- AGOSTINIS, C., BULLA, R., TRIPODO, C., GISMONDI, A., STABILE, H., BOSSI, F., GUARNOTTA, C., GARLANDA, C., DE SETA, F., SPESSOTTO, P., SANTONI, A., GHEBREHIWET, B., GIRARDI, G. & TEDESCO, F. 2010. An Alternative Role of C1q in Cell Migration and Tissue Remodeling: Contribution to Trophoblast Invasion and Placental Development. *Journal of Immunology*, 185, 4420-4429.
- AGRAWAL, A. & VOLANAKIS, J. E. 1994. Structural Determinants of the C1q-Binding Site of Human C-Reactive Protein (Crp). *Faseb Journal*, 8, A767-A767.
- ARLAUD, G. J., GABORIAUD, C., THIELENS, N. M., ROSSI, V., BERSCH, B., HERNANDEZ, J. F. & FONTECILLA-CAMPS, J. C. 2001. Structural biology of C1: dissection of a complex molecular machinery. *Immunological Reviews*, 180, 136-145.
- ASSIMEH, S. N. & PAINTER, R. H. 1975. Macromolecular Structure of 1st Component of Complement. *Journal of Immunology*, 115, 488-494.
- ALIMONTI, J. B., BALL, T. B. & FOWKE, K. R. 2003. Mechanisms of CD4(+) T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *Journal of General Virology*, 84, 1649-1661.
- ALIMONTI, J. B., BALL, T. B. & FOWKE, K. R. 2003. Mechanisms of CD4(+) T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *Journal of General Virology*, 84, 1649-1661.
- AUSTYN, J. M., STEINMAN, R. M., WEINSTEIN, D. E., GRANELLIPERNO, A. & PALLADINO, M. A. 1983. Dendritic Cells Initiate a 2-Stage Mechanism for Lymphocyte-T Proliferation. *Journal of Experimental Medicine*, 157, 1101-1115.
- BAILLY, Y., HAEBERLE, A. M., BLANQUET-GROSSARD, F., CHASSEROT-GOLAZ, S., GRANT, N., SCHULZE, T., BOMBARDE, G., GRASSI, J., CESBRON, J. Y. & LEMAIRE-VIEILLE, C. 2004. Prion protein (PrPc) immunocytochemistry and expression of the green fluorescent protein reporter gene under control of the bovine PrP gene promoter in the mouse brain. *Journal of Comparative Neurology*, 473, 244-269.
- BALZARINI, J. & VAN DAMME, L. 2007. Microbicide drug candidates to prevent HIV infection. *Lancet*, 369, 787-797.
- BALZARINI, J., VAN LAETHEM, K., DAELEMANS, D., HATSE, S., BUGATTI, A., RUSNATI, M., IGARASHI, Y., OKI, T. & SCHOLS, D. 2007. Pradimicin A, a carbohydrate-binding nonpeptidic lead compound for treatment of infections with viruses with highly glycosylated envelopes, such as human immunodeficiency virus. *Journal of Virology*, 81, 362-373.
- BANCHEREAU, J. & STEINMAN, R. M. 1998. Dendritic cells and the control of immunity. *Nature*, 392, 245-252.
- BARTHOLOMEW, R. M., ESSER, A. F. & EBERHARD, H. J. M. 1978. Lysis of Oncornaviruses by Human-Serum - Isolation of Viral Complement (C1) Receptor and Identification as P15e. *Journal of Experimental Medicine*, 147, 844-853.
- BARUAH, P., DUMITRIU, I. E., MALIK, T. H., COOK, H. T., DYSON, J., SCOTT, D., SIMPSON, E. & BOTTO, M. 2009. C1q enhances IFN-gamma production by antigen-specific T cells via the CD40 costimulatory pathway on dendritic cells. *Blood*, 113, 3485-3493.

- BASHIROVA, A. A., GEIJTENBEEK, T. B. H., VAN DUJNHOFEN, G. C. F., VAN VLIET, S. J., EILERING, J. B. G., MARTIN, M. P., WU, L., MARTIN, T. D., VIEBIG, N., KNOLLE, P. A., KEWALRAMANI, V. N., VAN KOOYK, Y. & CARRINGTON, M. 2001. A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. *Journal of Experimental Medicine*, 193, 671-678.
- BASU, S., BINDER, R. J., RAMALINGAM, T. & SRIVASTAVA, P. K. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity*, 14, 303-313.
- BELL, D., CHOMARAT, P., BROYLES, D., NETTO, G., HARB, G. M., LEBECQUE, S., VALLADEAU, J., DAVOUST, J., PALUCKA, K. A. & BANCHEREAU, J. 1999. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *Journal of Experimental Medicine*, 190, 1417-1425.
- BERUBE, N. G., SWANSON, X. H., BERTRAM, M. J., KITTLE, J. D., DIDENKO, V., BASKIN, D. S., SMITH, J. R. & PEREIRA-SMITH, O. M. 1999. Cloning and characterization of CRF, a novel C1q-related factor, expressed in areas of the brain involved in motor function. *Molecular Brain Research*, 63, 233-240.
- BEUTLER, B. 2004a. Inferences, questions and possibilities in toll-like receptor signalling. *Nature*, 430, 257-263.
- BEUTLER, B. 2004b. Innate immunity: an overview. *Molecular Immunology*, 40, 845-859.
- BHOOLA, K. D., FIGUEROA, C. D. & WORTHY, K. 1992. Bioregulation of Kinins - Kallikreins, Kininogens, and Kininases. *Pharmacological Reviews*, 44, 1-80.
- BIRO, A., THIELENS, N. M., CERVENAK, L., PROHASZKA, Z., FUST, G. & ARLAUD, G. J. 2007. Modified low density lipoproteins differentially bind and activate the C1 complex of complement. *Molecular Immunology*, 44, 1169-1177.
- BOGIN, O., KVANSAKUL, M., ROM, E., SINGER, J., YAYON, A. & HOHENESTER, E. 2002. Insight into Schmid metaphyseal chondrodysplasia from the crystal structure of the collagen X NC1 domain trimer. *Structure*, 10, 165-173.
- BOHNSACK, J. F., TENNER, A. J., LAURIE, G. W., KLEINMAN, H. K., MARTIN, G. R. & BROWN, E. J. 1985. C1q Binds to Laminin (Lam) - a Mechanism for Deposition and Retention of Immune-Complexes in Basement-Membranes (Bm). *Federation Proceedings*, 44, 1268-1268.
- BONAPARTE, R. S., HAIR, P. S., BANTHIA, D., MARSHALL, D. A., CUNNION, K. A. & KRISHNA, N. K. 2008. Human astrovirus coat protein inhibits serum complement activation via C1, the first component of the classical pathway. *Journal of Virology*, 82, 817-827.
- BORDIN, S., GHEBREHIWET, B. & PAGE, R. C. 1990. Participation of C1q and Its Receptor in Adherence of Human-Diploid Fibroblast. *Journal of Immunology*, 145, 2520-2526.
- BOSSI, F., FISCHETTI, F., REGOLI, D., DURIGUTTO, P., FROSSI, B., GOBEIL, F., GHEBREHIWET, B., PEERSCHKE, E. I., CICARDI, M. & TEDESCO, F. 2009. Novel pathogenic mechanism and therapeutic approaches to angioedema associated with C1 inhibitor deficiency. *Journal of Allergy and Clinical Immunology*, 124, 1303-1310.
- BOSSI, F., RIZZI, L., BULLA, R., TRIPODO, C., GUARNOTTA, C., NOVATI, F., GHEBREHIWET, B. & TEDESCO, F. 2011. C1q induces in vivo angiogenesis and promotes wound healing. *Molecular Immunology*, 48, 1676-1677.
- BRUNI, R. & ROIZMAN, B. 1996. Open reading frame P - A herpes simplex virus gene repressed during productive infection encodes a protein that binds a splicing factor

- and reduces synthesis of viral proteins made from spliced mRNA. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 10423-10427.
- BULLA, R., AGOSTINIS, C., BOSSI, F., RIZZI, L., DEBEUS, A., TRIPODO, C., RADILLO, O., DE SETA, F., GHEBREHIWET, B. & TEDESCO, F. 2008. Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. *Molecular Immunology*, 45, 2629-2640.
- BURTON, D. R., BOYD, J., BRAMPTON, A. D., EASTERBROOKSMITH, S. B., EMANUEL, E. J., NOVOTNY, J., RADEMACHER, T. W., VANSCHRAVENDIJK, M. R., STERNBERG, M. J. E. & DWEK, R. A. 1980. The C1q Receptor-Site on Immunoglobulin-G. *Nature*, 288, 338-344.
- CAO, W. P., BOBRY SHEV, Y. V., LORD, R. S. A., OAKLEY, R. E. I., LEE, S. H. & LU, J. H. 2003. Dendritic cells in the arterial wall express C1q: potential significance in atherogenesis. *Cardiovascular Research*, 60, 175-186.
- CARLAND, T. M. & GERWICK, L. 2010. The C1q domain containing proteins: Where do they come from and what do they do? *Developmental and Comparative Immunology*, 34, 785-790.
- CASTELLANO, G., SCHLAGWEIN, N., WOLTMAN, A. M., SCHENA, F. P., DAHA, M. R. & VAN KOOTEN, C. 2004. C1q is a potent regulator of human dendritic cell functions. *Molecular Immunology*, 41, 217-217.
- CASTELLANO, G., TROUW, L. A., FIORE, N., DAHA, M. R., SCHENA, F. P. & VAN KOOTEN, C. 2010. Infiltrating dendritic cells contribute to local synthesis of C1q in murine and human lupus nephritis. *Molecular Immunology*, 47, 2129-2137.
- CAFFREY, M. 2001. Model for the structure of the HIV gp41 ectodomain: insight into the intermolecular interactions of the gp41 loop. *Biochimica Et Biophysica Acta-Molecular Basis of Disease*, 1536, 116-122.
- CARAS, I. W., DAVITZ, M. A., RHEE, L., WEDDELL, G., MARTIN, D. W. & NUSSENZWEIG, V. 1987. Cloning of Decay-Accelerating Factor Suggests Novel Use of Splicing to Generate 2 Proteins. *Nature*, 325, 545-549.
- CHAN, D. C., FASS, D., BERGER, J. M. & KIM, P. S. 1997. Core structure of gp41 from the HIV envelope glycoprotein. *Cell*, 89, 263-273.
- CHEN, A., GADDIPATI, S., HONG, Y. Q., VOLKMAN, D. J., PEERSCHKE, E. I. B. & GHEBREHIWET, B. 1994. Human T-Cells Express Specific Binding-Sites for C1q - Role in T-Cell Activation and Proliferation. *Journal of Immunology*, 153, 1430-1440.
- CHEN, S. S. L., LEE, S. F. & WANG, C. T. 2001. Cellular membrane-binding ability of the C-terminal cytoplasmic domain of human immunodeficiency virus type 1 envelope transmembrane protein gp41. *Journal of Virology*, 75, 9925-9938.
- CHOI, Y., KWON, Y. C., KIM, S. I., PARK, J. M., LEE, Y. H. & AHN, B. Y. 2009. A hantavirus causing hemorrhagic fever with renal syndrome requires gC1qR/p32 for efficient cell binding and infection (vol 381, pg 178, 2008). *Virology*, 384, 253-253.
- COLMAN, R. W., PIXLEY, R. A., NAJAMUNNISA, S., YAN, W. Y., WANG, J. Y., MAZAR, A. & MCCRAE, K. R. 1997. Binding of high molecular weight kininogen to human endothelial cells is mediated via a site within domains 2 and 3 of the urokinase receptor. *Journal of Clinical Investigation*, 100, 1481-1487.
- COOPER, N. R. 1985. The Classical Complement Pathway - Activation and Regulation of the 1st Complement Component. *Advances in Immunology*, 37, 151-216.
- CSAKO, G., SUBA, E. A. & HERP, A. 1981. Similarities and Dissimilarities between the Binding Ability of Clq and Collagen. *Clinical and Experimental Immunology*, 44, 181-190.

- CUMMINGS, K. L., ROSEN, H. R. & HAHN, Y. S. 2009. Frequency of gC1qR(+)CD4(+) T cells increases during acute hepatitis C virus infection and remains elevated in patients with chronic infection. *Clinical Immunology*, 132, 401-411.
- CURTIS, B. M., SCHARNOWSKE, S. & WATSON, A. J. 1992. Sequence and Expression of a Membrane-Associated C-Type Lectin That Exhibits Cd4-Independent Binding of Human-Immunodeficiency-Virus Envelope Glycoprotein-Gp120. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 8356-8360.
- CUTLER, A. J., CORNALL, R. J., FERRY, H., MANDERSON, A. P., BOTTO, M. & WALPORT, M. J. 2001. Intact B cell tolerance in the absence of the first component of the classical complement pathway. *European Journal of Immunology*, 31, 2087-2093.
- DAFFERN, P. J., PFEIFER, P. H., EMBER, J. A. & HUGLI, T. E. 1995. C3a Is a Chemotaxin for Human Eosinophils but Not for Neutrophils .1. C3a Stimulation of Neutrophils Is Secondary to Eosinophil Activation. *Journal of Experimental Medicine*, 181, 2119-2127.
- DE FOUGEROLLES, A. R., SPRAGUE, A. G., NICKERSON-NUTTER, C. L., CHI-ROSSO, G., RENNERT, P. D., GARDNER, H., GOTWALS, P. J., LOBB, R. R. & KOTELIANSKY, V. E. 2000. Regulation of inflammation by collagen-binding integrins alpha 1 beta 1 and alpha 2 beta 1 in models of hypersensitivity and arthritis. *Journal of Clinical Investigation*, 105, 721-729.
- DENDORFER, A., WOLFRUM, S., WAGEMANN, M., QADRI, F. & DOMINIAK, P. 2001. Pathways of bradykinin degradation in blood and plasma of normotensive and hypertensive rats. *American Journal of Physiology-Heart and Circulatory Physiology*, 280, H2182-H2188.
- DEDIO, J., JAHNEN-DECHENT, W., BACHMANN, M. & MULLER-ESTERL, W. 1998. The multiligand-binding protein gC1qR, putative Clq receptor, is a mitochondrial protein. *Journal of Immunology*, 160, 3534-3542.
- DOLIANA, R., BOT, S., MUNGIGUERRA, G., CANTON, A., CILLI, S. P. & COLOMBATTI, A. 2001. Isolation and characterization of EMILIN-2, a new component of the growing EMILINs family and a member of the EMI domain-containing superfamily. *Journal of Biological Chemistry*, 276, 12003-12011.
- DOLIANA, R., MONGIAT, M., BUCCIOTTI, F., GIACOMELLO, E., DEUTZMANN, R., VOLPIN, D., BRESSAN, G. M. & COLOMBATTI, A. 1999. EMILIN, a component of the elastic fiber and a new member of the C1q/Tumor necrosis factor superfamily of proteins. *Journal of Biological Chemistry*, 274, 16773-16781.
- DORFEL, D., APPEL, S., GRUNEBACH, F., WECK, M. M., MULLER, M. R., HEINE, A. & BROSSART, P. 2005. Processing and presentation of HLA class I and II epitopes by dendritic cells after transfection with in vitro-transcribed MUC1 RNA. *Blood*, 105, 3199-3205.
- DUBLET, B., VERNET, T. & VAN DER REST, M. 1999. Schmid's metaphyseal chondrodysplasia mutations interfere with folding of the C-terminal domain of human collagen X expressed in *Escherichia coli*. *Journal of Biological Chemistry*, 274, 18909-18915.
- DUNKELBERGER, J. R. & SONG, W. C. 2010. Complement and its role in innate and adaptive immune responses. *Cell Research*, 20, 34-50.
- DUUS, K., HANSEN, E. W., TACNET, P., FRACHET, P., ARLAUD, G. J., THIELENS, N. M. & HOUEN, G. 2010. Direct interaction between CD91 and C1q. *Febs Journal*, 277, 3526-3537.

- EBENBICHLER, C. F., THIELENS, N. M., VORNHAGEN, R., MARSCHANG, P., ARLAUD, G. J. & DIERICH, M. P. 1991. Human-Immunodeficiency-Virus Type-1 Activates the Classical Pathway of Complement by Direct C1-Binding through Specific Sites in the Transmembrane Glycoprotein-Gp41. *Journal of Experimental Medicine*, 174, 1417-1424
- ECHTENACHER, B., MANNEL, D. N. & HULTNER, L. 1996. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature*, 381, 75-77.
- EDELSON, B. T., STRICKER, T. P., LI, Z. Z., DICKESON, S. K., SHEPHERD, V. L., SANTORO, S. A. & ZUTTER, M. M. 2006. Novel collectin/C1q receptor mediates mast cell activation and innate immunity. *Blood*, 107, 143-150.
- EGGLETON, P., GHEBREHIWET, B., SASTRY, K. N., COBURN, J. P., ZANER, K. S., REID, K. B. M. & TAUBER, A. I. 1995. Identification of a Gc1q-Binding Protein (Gc1q-R) on the Surface of Human Neutrophils - Subcellular-Localization and Binding-Properties in Comparison with the Cc1q-R. *Journal of Clinical Investigation*, 95, 1569-1578.
- EGGLETON, P., REID, K. B. M. & TENNER, A. J. 1998. C1q - how many functions? How many receptors? *Trends in Cell Biology*, 8, 428-431.
- ELLGAARD, L., RIEK, R., FRICKEL, E., HERRMANN, T., BRAUN, D., GUNTERT, P., WUTHRICH, K. & HELENIUS, A. 2001. NMR structure of the calreticulin P-domain: Implications for chaperone function. *Molecular Biology of the Cell*, 12, 132a-132a.
- ENGELMAN, J. A., LUO, J. & CANTLEY, L. C. 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nature Reviews Genetics*, 7, 606-619.
- ERLICH, P., DUMESTRE-PERARD, C., LING, W. L., LEMAIRE-VIEILLE, C., SCHOEHN, G., ARLAUD, G. J., THIELENS, N. M., GAGNON, J. & CESBRON, J. Y. 2010. Complement Protein C1q Forms a Complex with Cytotoxic Prion Protein Oligomers. *Journal of Biological Chemistry*, 285, 19267-19276.
- FARBER, K., CHEUNG, G., MITCHELL, D., WALLIS, R., WEIHE, E., SCHWAEBLE, W. & KETTENMANN, H. 2009. C1q, the Recognition Subcomponent of the Classical Pathway of Complement, Drives Microglial Activation. *Journal of Neuroscience Research*, 87, 644-652.
- FAUSTHER-BOVENDO, H., VIEILLARD, V., SAGAN, S., BISMUTH, G. & DEBRE, P. 2010. HIV gp41 Engages gC1qR on CD4+T Cells to Induce the Expression of an NK Ligand through the PIP3/H2O2 Pathway. *Plos Pathogens*, 6.
- FENG, X. D., TONNESEN, M. G., PEERSCHKE, E. I. B. & GHEBREHIWET, B. 2002. Cooperation of C1q receptors and integrins in C1q-mediated endothelial cell adhesion and spreading. *Journal of Immunology*, 168, 2441-2448.
- FERRY, H., POTTER, P. K., CROCKFORD, T. L., NIJNIK, A., EHRENSTEIN, M. R., WALPORT, M. J., BOTTO, M. & CORNALL, R. J. 2007. Increased positive selection of B1 cells and reduced B cell tolerance to intracellular antigens in C1q-deficient mice. *Journal of Immunology*, 178, 2916-2922.
- FINKEL, T. H., RIZVI, T., CASELLA, C. R., SATTERFIELD, W., COTTON, M. F., RAPAPORT, E. L., BABA, T. W. & RUPRECHT, R. M. 1995. Apoptosis in Acute and Chronic Hiv SIV Infection. *Aids Research and Human Retroviruses*, 11, S78-S78.
- FRASER, D. A., PISALYAPUT, K. & TENNER, A. J. 2010. C1q enhances microglial clearance of apoptotic neurons and neuronal blebs, and modulates subsequent inflammatory cytokine production. *Journal of Neurochemistry*, 112, 733-743.

- FREY, G., CHEN, J., RITS-VOLLOCH, S., FREEMAN, M. M., ZOLLA-PAZNER, S. & CHEN, B. 2010. Distinct conformational states of HIV-1 gp41 are recognized by neutralizing and non-neutralizing antibodies. *Nature Structural & Molecular Biology*, 17, 1486-U118.
- FREY, G., CHEN, J., RITS-VOLLOCH, S., FREEMAN, M. M., ZOLLA-PAZNER, S. & CHEN, B. 2010. Distinct conformational states of HIV-1 gp41 are recognized by neutralizing and non-neutralizing antibodies. *Nature Structural & Molecular Biology*, 17, 1486-U118.
- 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. *Journal of Biological Chemistry*, 278, 46974-46982.
- GADJEVA, M., ROUMENINA, L., ZLATAROVA, A., TSACHEVA, I., REID, K. B. M., KISHORE, U. & KOJOUHAROVA, M. 2004. Molecular mechanisms of recognition between complement component C1q globular domain and its specific ligands. *Molecular Immunology*, 41, 232-232.
- GADJEVA, M., ZLATAROVA, A., RUSEVA, M., KISHORE, U. & KOJOUHAROVA, M. 2010. Mutational analysis of ligand-binding activities of recombinant gC1q heterotrimer. *Molecular Immunology*, 47, 2260-2260.
- GEHLENBORG, N., HWANG, D., LEE, I. Y., YOO, H., BAXTER, D., PETRITIS, B., PITSTICK, R., MARZOLF, B., DEARMOND, S. J., CARLSON, G. A. & HOOD, L. 2009. The Prion Disease Database: a comprehensive transcriptome resource for systems biology research in prion diseases. *Database-the Journal of Biological Databases and Curation*.
- GEIJTENBEEK, T. B. H., KROOSHOO, D. J. E. B., BLEIJS, D. A., VAN VLIET, S. J., VAN DUJINHOVEN, G. C. F., GRABOVSKY, V., ALON, R., FIGDOR, C. G. & VAN KOOYK, Y. 2000a. DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. *Nature Immunology*, 1, 353-357.
- GEIJTENBEEK, T. B. H., KWON, D. S., TORENSMA, R., VAN VLIET, S. J., VAN DUJINHOVEN, G. C. F., MIDDEL, J., CORNELISSEN, I. L. M. H. A., NOTTET, H. S. L. M., KEWALRAMANI, V. N., LITTMAN, D. R., FIGDOR, C. G. & VAN KOOYK, Y. 2000b. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell*, 100, 587-597.
- GEIJTENBEEK, T. B. H., TORENSMA, R., VAN VLIET, S. J., VAN DUJINHOVEN, G. C. F., ADEMA, G. J., VAN KOOYK, Y. & FIGDOR, C. G. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*, 100, 575-585..
- GERWICK, L., REYNOLDS, W. S. & BAYNE, C. J. 2000. A precerebellin-like protein is part of the acute phase response in rainbow trout, *Oncorhynchus mykiss*. *Developmental and Comparative Immunology*, 24, 597-607.
- GHAI, R., WATERS, P., ROUMENINA, L. T., GADJEVA, M., KOJOUHAROVA, M. S., REID, K. B. M., SIM, R. B. & KISHORE, U. 2007. C1q and its growing family. *Immunobiology*, 212, 253-266.
- GHEBREHIWET, B, JESTY J, VINAYAGASUNDARAM R, VINAYAGASUNDARAM U, JI Y, VALENTINO A, TUMMA N, HOSSZU KK, AND PEERSCHKE EIB. 2013. Targeting gC1qR domains for therapy against infection and inflammation. *Complement Therapeutics in Advances in Exp Med Biol*. 734:97-110
- GHEBREHIWET, B, JESTY J, XU, S, VINAYAGASUNDARAM R, VINAYAGASUNDARAM U, JI Y, VALENTINO A HOSSZU KK, MATTHEW, S, JOSEPH, K, KAPLAN A.P AND PEERSCHKE EIB, 2011 Structure-Function Studies using deletion mutants identify domains of

- gC1qR/P33 as potential therapeutic targets for vascular permeability and inflammation, *Frontiers In Immunology*
- GHEBREHIWET, B. & PEERSCHKE, E. I. B. 1998. Structure and function of gC1q-R: a multiligand binding cellular protein. *Immunobiology*, 199, 225-238.
- GHEBREHIWET, B., HABICHT, G. S. & BECK, G. 1990. Interaction of C1q with Its Receptor on Cultured-Cell Lines Induces an Anti-Proliferative Response. *Clinical Immunology and Immunopathology*, 54, 148-160.
- GHEBREHIWET, B., JESTY, J. & PEERSCHKE, E. I. B. 2002. GC1q-R/p33: Structure-function predictions from the crystal structure. *Immunobiology*, 205, 421-432.
- GHEBREHIWET, B., KEW, R. R., GRUBER, B. L., MARCHESE, M. J., PEERSCHKE, E. I. B. & REID, K. B. M. 1995. Murine Mast-Cell Clq Receptors Are Involved in the Induction of Chemotaxis and Chemokinesis. *Faseb Journal*, 9, A776-A776.
- GHEBREHIWET, B., LIM, B. L., KUMAR, R., FENG, X. D. & PEERSCHKE, E. I. B. 2001. gClq-R/p33, a member of a new class of multifunctional and multicompartmental cellular proteins, is involved in inflammation and infection. *Immunological Reviews*, 180, 65-77.
- GHEBREHIWET, B., LIM, B. L., PEERSCHKE, E. I. B., WILLIS, A. C. & REID, K. B. M. 1994. Isolation, Cdna Cloning, and Overexpression of a 33-Kd Cell-Surface Glycoprotein That Binds to the Globular Heads of C1q. *Journal of Experimental Medicine*, 179, 1809-1821.
- GHEBREHIWET, B., PETERSON, K., PEERSCHKE, E. I. B., REDDIGARI, S. R. & REID, K. B. M. 1994. Purification and Immunochemical Characterization of Soluble Forms of the 2 Types of C1q Receptors - Cc1q-R and Gc1q-R. *Faseb Journal*, 8, A475-A475.
- GHEBREHIWET, B., TANTRAL, L., TITMUS, M. A., PANESSA-WARREN, B. J., TORTORA, G. T., WONG, S. S. & WARREN, J. B. 2007. The exosporium of *B.cereus* contains a binding site for gC1qR/p33: Implication in spore attachment and/or entry. *Current Topics in Innate Immunity*, 598, 181-197.
- GIGLI, I., FUJITA, T. & NUSSENZWEIG, V. 1979. Modulation of the Classical Pathway C-3 Convertase by Plasma-Proteins C-4 Binding-Protein and C3b Inactivator. *Proceedings of the National Academy of Sciences of the United States of America*, 76, 6596-6600.
- GOODMAN, E. B., ANDERSON, D. C. & TENNER, A. J. 1995. C1q Triggers Neutrophil Superoxide Production by a Unique Cd18-Dependent Mechanism. *Journal of Leukocyte Biology*, 58, 168-176.
- GREENLEE, M. C., SULLIVAN, S. A. & BOHLSON, S. S. 2009. Detection and characterization of soluble CD93 released during inflammation. *Inflammation Research*, 58, 909-919.
- GREGORY, C. D. & DEVITT, A. 2004. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology*, 113, 1-14.
- GROENEVELD, T. W. L., OROSZLAN, M., OWENS, R. T., FABER-KROL, M. C., BAKKER, A. C., ARLAUD, G. J., MCQUILLAN, D. J., KISHORE, U., DAHA, M. R. & ROOS, A. 2005. Interactions of the extracellular matrix proteoglycans decorin and biglycan with C1q and collectins. *Journal of Immunology*, 175, 4715-4723.
- GROENEVELD, T. W. L., RAMWADHDOEBE, T. H., TROUW, L. A., VAN DEN HAM, D. L., VAN DER BORDEN, V., DRIJFHOUT, J. W., HIEMSTRA, P. S., DAHA, M. R. & ROOS, A. 2007. Human neutrophil peptide-1 inhibits both the classical and the lectin pathway of complement activation. *Molecular Immunology*, 44, 3608-3614.

- GUAN, E., ROBINSON, S. L., GOODMAN, E. B. & TENNER, A. J. 1994. Cell-Surface Protein Identified on Phagocytic-Cells Modulates the C1q-Mediated Enhancement of Phagocytosis. *Journal of Immunology*, 152, 4005-4016.
- GUO, N., WEREMOWICZ, S., LYNCH, N., LIM, B. L., SCHWAEBLE, W., PEERSCHKE, E. I. B., MORTON, C. C., REID, K. B. M., GHEBREHIWET, B. & SASTRY, K. N. 1997. Assignment of C1QBP encoding the C1q globular domain binding protein (gC1q-R) to human chromosome 17 band p13.3 by in situ hybridization. *Cytogenetics and Cell Genetics*, 77, 283-284.
- GUO, W. X., GHEBREHIWET, B., WEKSLER, B., SCHWEITZER, K. & PEERSCHKE, E. I. B. 1999. Up-regulation of endothelial cell binding proteins receptors for complement component C1q by inflammatory cytokines. *Journal of Laboratory and Clinical Medicine*, 133, 541-550.
- GUO, Y., COLMAN, R. W., 2005, Two faces of high molecular weight kininogen (HK) in angiogenesis: bradykinin turns it on and cleaved HK (HKa) turns it off, 2005, *Throm. Haemost*, 670-676
- HAFFAR, O. K., DOWBENKO, D. J. & BERMAN, P. W. 1988. Topogenic Analysis of the Human Immunodeficiency Virus Type-1 Envelope Glycoprotein, Gp160, in Microsomal-Membranes. *Journal of Cell Biology*, 107, 1677-1687.
- HASAN, A. A. K., ZISMAN, T. & SCHMAIER, A. H. 1998. Identification of cytokeratin 1 as a binding protein and presentation receptor for kininogens on endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 3615-3620.
- HAYWARD, C. P. M. & KELTON, J. G. 1995. Multimerin - a Multimeric Protein Stored in Platelet Alpha-Granules. *Platelets*, 6, 1-10.
- HERNANDEZPANDO, R. & ROOK, G. A. W. 1994. The Role of Tnf-Alpha in T-Cell-Mediated Inflammation Depends on the Th1/Th2 Cytokine Balance. *Immunology*, 82, 591-595.
- HERWALD, H., DEDIO, J., KELLNER, R., LOOS, M. & MULLERESTERL, W. 1996. Isolation and characterization of the kininogen-binding protein p33 from endothelial cells - Identity with the gC1q receptor. *Journal of Biological Chemistry*, 271, 13040-13047.
- HESS, J. F., BORKOWSKI, J. A., YOUNG, G. S., STRADER, C. D. & RANSOM, R. W. 1992. Cloning and Pharmacological Characterization of a Human Bradykinin (Bk-2) Receptor. *Biochemical and Biophysical Research Communications*, 184, 260-268.
- HEYWORTH, P. G., KNAUS, U. G., SETTLEMAN, J., CURNUTTE, J. T. & BOKOCH, G. M. 1993. Regulation of NADPH Oxidase Activity by Rac GTPase-Activating Protein(S). *Molecular Biology of the Cell*, 4, 1217-1223.
- HONG, Q. Y., SZE, C. I., LIN, S. R., LEE, M. H., HE, R. Y., SCHULTZ, L., CHANG, J. Y., CHEN, S. J., BOACKLE, R. J., HSU, L. J. & CHANG, N. S. 2009. Complement C1q Activates Tumor Suppressor WWOX to Induce Apoptosis in Prostate Cancer Cells. *Plos One*, 4.
- HOORELBEKE, B., XUE, J., LIWANG, P. J. & BALZARINI, J. 2013. Role of the Carbohydrate-Binding Sites of Griffithsin in the Prevention of DC-SIGN-Mediated Capture and Transmission of HIV-1. *Plos One*, 8.
- HOPFER, U., FUKAI, N., HOPFER, H., WOLF, G., JOYCE, N., LI, E. & OLSEN, B. R. 2005. Targeted disruption of Col8a1 and Col8a2 genes in mice leads to anterior segment abnormalities in the eye. *Faseb Journal*, 19, 1232-1244.
- HOSSZU, K. K., VALENTINO, A., VINAYAGASUNDARAM, U., VINAYAGASUNDARAM, R., JOYCE, M. G., JI, Y., PEERSCHKE, E. I. B. & GHEBREHIWET, B. 2012. DC-SIGN, C1q, and gC1qR

- form a trimolecular receptor complex on the surface of monocyte-derived immature dendritic cells. *Blood*, 120, 1228-1236.
- HOSSZU, K. K., VALENTINO, A., VINAYAGASUNDARAM, U., VINAYAGASUNDARAM, R., JOYCE, M. G., JI, Y., PEERSCHKE, E. I. B. & GHEBREHIWET, B. 2012. DC-SIGN, C1q, and gC1qR form a trimolecular receptor complex on the surface of monocyte-derived immature dendritic cells. *Blood*, 120, 1228-1236.
- HOSSZU, K. K., VINAYAGASUNDARAM, U., HABEL, D., JI, Y., VINAYAGASUNDARAM, R., PEERSCHKE, E. I. B. & GHEBREHIWET, B. 2010. Evidence that the full-length gC1qR (residues 1-282) is expressed on the cell surface. *Molecular Immunology*, 47, 2223-2223.
- HOSSZU, K., SANTIAGO-SCHWARZ, F., PEERSCHKE, E. & GHEBREHIWET, B. 2008. C1q is a molecular switch that regulates dendritic cell maturation at the monocyte-to-dendritic cell transition. *Molecular Immunology*, 45, 4142-4143.
- IWASAKI, A. & MEDZHITOV, R. 2010. Regulation of Adaptive Immunity by the Innate Immune System. *Science*, 327, 291-295.
- JANEWAY, C. A. & MEDZHITOV, R. 2002. Innate immune recognition. *Annual Review of Immunology*, 20, 197-216.
- JANEWAY, C. A., DIANZANI, U., PORTOLES, P., RATH, S., REICH, E. P., ROJO, J., YAGI, J. & MURPHY, D. B. 1989. Cross-Linking and Conformational Change in T-Cell Receptors - Role in Activation and in Repertoire Selection. *Immunological Recognition*, Pts 1 and 2, 54, 657-666.
- JANEWAY, CA, JR, TRAVERS, P, WALPORT, M, 2001, The complement system and Innate Immunity, *Immunobiology: The Immune System In Health and Disease*. New York: Garland Science
- JIANG, J. Z., ZHANG, Y., KRAINER, A. R. & XU, R. M. 1999. Crystal structure of human p32, a doughnut-shaped acidic mitochondrial matrix protein. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 3572-3577.
- JOSEPH, K. & KAPLAN, A. P. 2005. Formation of bradykinin: A major contributor to the innate inflammatory response. *Advances in Immunology*, Vol 86, 86, 159-208.
- JOSEPH, K., GHEBREHIWET, B. & KAPLAN, A. P. 1999. Cytokeratin 1 and gC1qR mediate high molecular weight kininogen binding to endothelial cells. *Clinical Immunology*, 92, 246-255.
- JOSEPH, K., GHEBREHIWET, B., PEERSCHKE, E. I. B., REID, K. B. M. & KAPLAN, A. P. 1996. Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: Identity with the receptor that binds to the globular "heads" of C1q (gC1q-R). *Proceedings of the National Academy of Sciences of the United States of America*, 93, 8552-8557.
- JOSEPH, K., THOLANIKUNNEL, B. G., GHEBREHIWET, B. & KAPLAN, A. P. 2004. Interaction of high molecular weight kininogen binding proteins on endothelial cells. *Thrombosis and Haemostasis*, 91, 61-70.
- KAISHO, T., TAKEUCHI, O., KAWAI, T., HOSHINO, K. & AKIRA, S. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *Journal of Immunology*, 166, 5688-5694.
- KALIA, V., SARKAR, S., GUPTA, P. & MONTELARO, R. C. 2003. Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common

- functions in cell-cell fusion but distinct roles in virion envelope incorporation. *Journal of Virology*, 77, 3634-3646.
- KANG, Y. S., DO, Y., LEE, H. K., PARK, S. H., CHEONG, C., LYNCH, R. M., LOEFFLER, J. M., STEINMAN, R. M. & PARK, C. G. 2006. A dominant complement fixation pathway for pneumococcal polysaccharides initiated by SIGN-R1 interacting with C1q. *Cell*, 125, 47-58.
- KANG, Y. S., YAMAZAKI, S., IYODA, T., PACK, M., BRUENING, S. A., KIM, J. Y., TAKAHARA, K., INABA, K., STEINMAN, R. M. & PARK, C. G. 2003. SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran. *International Immunology*, 15, 177-186.
- KAPLAN, A. P. 2002. Chronic urticaria and angioedema. *New England Journal of Medicine*, 346, 175-179.
- KAPLAN, A. P., JOSEPH, K., SHIBAYAMA, Y., REDDIGARI, S. & GHEBREHIWET, B. 2001. Activation of the plasma kinin forming cascade along cell surfaces. *International Archives of Allergy and Immunology*, 124, 339-342.
- KENNETT RH, MCKEARN TJ AND BECHTOL KB 1980. Enzyme linked antibody assay with cells attached to polyvinyl chloride plates. plenum press, New York
- KHAN, M. M., BRADFORD, H. N., ISORDIA-SALAS, I., LIU, Y. C., WU, Y., ESPINOLA, R. G., GHEBREHIWET, B. & COLMAN, R. W. 2006. High-molecular-weight kininogen fragments stimulate the secretion of cytokines and chemokines through uPAR, Mac-1, and gC1qR in monocytes. *Arteriosclerosis Thrombosis and Vascular Biology*, 26, 2260-2266.
- KIM, K. B., KIM, B. W., CHOO, H. J., KWON, Y. C., AHN, B. Y., CHOI, J. S., LEE, J. S. & KO, Y. G. 2009. Proteome analysis of adipocyte lipid rafts reveals that gC1qR plays essential roles in adipogenesis and insulin signal transduction. *Proteomics*, 9, 2373-2382.
- KISHORE, U. & REID, K. B. M. 1999. Modular organization of proteins containing Clq-like globular domain. *Immunopharmacology*, 42, 15-21.
- KISHORE, U. & REID, K. B. M. 2000. C1q: structure, function, and receptors. *Immunopharmacology*, 49, 159-170.
- KISHORE, U., GABORIAUD, C., WATERS, P., SHRIVE, A. K., GREENHOUGH, T. J., REID, K. B. M., SIM, R. B. & ARLAUD, G. J. 2004a. C1q and tumor necrosis factor superfamily: modularity and versatility. *Trends in Immunology*, 25, 551-561.
- KISHORE, U., GUPTA, S. K., PERDIKOULIS, M. V., KOJOUHAROVA, M. S., URBAN, B. C. & REID, K. B. M. 2003. Modular organization of the carboxyl-terminal, globular head region of human C1q A, B, and C chains. *Journal of Immunology*, 171, 812-820.
- KISHORE, U., KOJOUHAROVA, M. S. & REID, K. B. M. 2002. Recent progress in the understanding of the structure-function relationships of the globular head regions of C1q. *Immunobiology*, 205, 355-364.
- KISHORE, U., LEIGH, L. E. A., EGGLETON, P., STRONG, P., PERDIKOULIS, M. V., WILLIS, A. C. & REID, K. B. M. 1998. Functional characterization of a recombinant form of the C-terminal, globular head region of the B-chain of human serum complement protein, C1q. *Biochemical Journal*, 333, 27-32.
- KISHORE, U., PERDIKOULIS, M. V., STRONG, P. & REID, K. B. M. 1998. A homotrimer of the B-chain globular head region of C1q is an inhibitor of C1q-mediated complement activation. *Molecular Immunology*, 35, 375-375.
- KISHORE, U., SONTHEIMER, R. D., SASTRY, K. N., ZANER, K. S., ZAPPI, E. G., HUGHES, G. R. V., KHAMASHTA, M. A., STRONG, P., REID, K. B. M. & EGGLETON, P. 1997. Release of

- calreticulin from neutrophils may alter C1q-mediated immune functions. *Biochemical Journal*, 322, 543-550.
- KISHORE, U., STRONG, P., PERDIKOULIS, M. V. & REID, K. B. M. 2001. A recombinant homotrimer, composed of the alpha helical neck region of human surfactant protein D and C1q B chain globular domain, is an inhibitor of the classical complement pathway. *Journal of Immunology*, 166, 559-565.
- KISHORE, W., GHAI, R., GREENHOUGH, T. J., SHRIVE, A. K., BONIFATI, D. M., GADJEVA, M. G., WATERS, P., KOJOUHAROVA, M. S., CHAKRABORTY, T. & AGRAWAL, A. 2004b. Structural and functional anatomy of the globular domain of complement protein C1q. *Immunology Letters*, 95, 113-128.
- KITTESEN, D. J., CHIANESE-BULLOCK, K. A., YAO, Z. Q., BRACIALE, T. J. & HAHN, Y. S. 2000. Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *Journal of Clinical Investigation*, 106, 1239-1249.
- KLEIN, M. A., KAESER, P. S., SCHWARZ, P., WEYD, H., XENARIOS, I., ZINKERNAGEL, R. M., CARROLL, M. C., VERBEEK, J. S., BOTTO, M., WALPORT, M. J., MOLINA, H., KALINKE, U., ACHA-ORBEA, H. & AGUZZI, A. 2001. Complement facilitates early prion pathogenesis. *Nature Medicine*, 7, 488-492.
- KOJOUHAROVA, M. S., GADJEVA, M. G., TSACHEVA, I. G., ZLATAROVA, A., ROUMENINA, L. T., TCHORBADJIEVA, M. I., ATANASOV, B. P., WATERS, P., URBAN, B. C., SIM, R. B., REID, K. B. M. & KISHORE, U. 2004. Mutational analyses of the recombinant globular regions of human C1q A, B, and C chains suggest an essential role for arginine and histidine residues in the C1q-IgG interaction. *Journal of Immunology*, 172, 4351-4358.
- KOJOUHAROVA, M. S., TSACHEVA, I. G., TCHORBADJIEVA, M. I., REID, K. B. M. & KISHORE, U. 2003. Localization of ligand-binding sites on human C1q globular head region using recombinant globular head fragments and single-chain antibodies. *Biochimica Et Biophysica Acta-Proteins and Proteomics*, 1652, 64-74.
- KONDO, H., SHIMOMURA, I., MATSUKAWA, Y., KUMADA, M., TAKAHASHI, M., MATSUDA, M., OUCHI, N., KIHARA, S., KAWAMOTO, T., SUMITSUJI, S., FUNAHASHI, T. & MATSUZAWA, Y. 2002. Association of adiponectin mutation with type 2 diabetes - A candidate gene for the insulin resistance syndrome. *Diabetes*, 51, 2325-2328.
- KOROTZER, A. R., WATT, J., CRIBBS, D., TENNER, A. J., BURDICK, D., GLABE, C. & COTMAN, C. W. 1995. Cultured Rat Microglia Express C1q and Receptor for C1q - Implications for Amyloid Effects on Microglia. *Experimental Neurology*, 134, 214-221.
- KOUSER, L., ABDUL-AZIZ, M., NAYAK, A., STOVER, C. M., SIM, R. B. & KISHORE, U. 2013. Properdin and factor h: opposing players on the alternative complement pathway "see-saw". *Front Immunol*, 4, 93
- KOVACS, H., CAMPBELL, I. D., STRONG, P., JOHNSON, S., WARD, F. J., REID, K. B. M. & EGGLETON, P. 1998. Evidence that C1q binds specifically to C(H)2-like immunoglobulin gamma motifs present in the autoantigen calreticulin and interferes with complement activation. *Biochemistry*, 37, 17865-17874.
- KREMLITZKA, M., POLGAR, A., FULOP, L., KISS, E., POOR, G. & ERDEI, A. 2013. Complement receptor type 1 (CR1, CD35) is a potent inhibitor of B-cell functions in rheumatoid arthritis patients. *International Immunology*, 25, 25-33.
- KRUMDIECK, R., HOOK, M., ROSENBERG, L. C. & VOLANAKIS, J. E. 1992. The Proteoglycan Decorin Binds C1q and Inhibits the Activity of the C1 Complex. *Journal of Immunology*, 149, 3695-3701.

- KUNA, P., IYER, M., PEERSCHKE, E. I. B., KAPLAN, A. P., REID, K. B. M. & GHEBREHIWET, B. 1996. Human C1q induces eosinophil migration. *Clinical Immunology and Immunopathology*, 81, 48-54.
- KUOPPALA, A., LINDSTEDT, K. A., SAARINEN, J., KOVANEN, P. T. & KOKKONEN, J. O. 2000. Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma. *American Journal of Physiology-Heart and Circulatory Physiology*, 278, H1069-H1074.
- KVANSAKUL, M., BOGIN, O., HOHENESTER, E. & YAYON, A. 2003. Crystal structure of the collagen alpha 1 (VIII) NC1 trimer. *Matrix Biology*, 22, 145-152.
- LACY, P. & STOW, J. L. 2011. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood*, 118, 9-18.
- LAEMMLI, U. K. 1970. Cleavage of Structural Proteins during Assembly of Head of Bacteriophage-T4. *Nature*, 227, 680-&.
- LASSER, G., GUCHHAIT, P., ELLSWORTH, J. L., SHEPPARD, P., LEWIS, K., BISHOP, P., CRUZ, M. A., LOPEZ, J. A. & FRUEBIS, J. 2006. C1qTNF-related protein-1 (CTRP-1): a vascular wall protein that inhibits collagen-induced platelet aggregation by blocking VWF binding to collagen. *Blood*, 107, 423-430.
- LEADBETTER, E. A., RIFKIN, I. R., HOHLBAUM, A. M., BEAUDETTE, B. C., SHLOMCHIK, M. J. & MARSHAK-ROTHSTEIN, A. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*, 416, 603-607.
- LEAVY, O. 2006. A new way to get complement. *Nature Reviews Microbiology*, 4, 490-491.
- LEE, S. F., KO, C. Y., WANG, C. T. & CHEN, S. S. L. 2002. Effect of point mutations in the N terminus of the lentivirus lytic peptide-1 sequence of human immunodeficiency virus type 1 transmembrane protein gp41 Env stability. *Journal of Biological Chemistry*, 277, 15363-15375.
- LEE, S. F., WANG, C. T., LIANG, J. Y. P., HONG, S. L., HUANG, C. C. & CHEN, S. S. L. 2000. Multimerization potential of the cytoplasmic domain of the human immunodeficiency virus type 1 transmembrane glycoprotein gp41. *Journal of Biological Chemistry*, 275, 15809-15819.
- LEEB-LUNDBERG, L. M. F., KANG, D. S., LAMB, D. S., LAMB, M. E. & FATHY, D. B. 2001. The human B1 bradykinin receptor exhibits high ligand-independent, constitutive activity - Roles of residues in the fourth intracellular and third transmembrane domains. *Journal of Biological Chemistry*, 276, 8785-8792.
- LEGLER, D. F., LOETSCHER, M., JONES, S. A., DAHINDEN, C. A., AROCK, M. & MOSER, B. 1996. Expression of high- and low-affinity receptors for C3a on the human mast cell line, HMC-1. *European Journal of Immunology*, 26, 753-758.
- LEIGH, L. E. A., GHEBREHIWET, B., PERERA, T. P. S., BIRD, I. N., STRONG, P., KISHORE, U., REID, K. B. M. & EGGLETON, P. 1998. C1q-mediated chemotaxis by human neutrophils: involvement of gC1qR and G-protein signalling mechanisms. *Biochemical Journal*, 330, 247-254.
- LIM, B. L., REID, K. B. M., GHEBREHIWET, B., PEERSCHKE, E. I. B., LEIGH, L. A. E. & PREISSNER, K. T. 1996. The binding protein for globular heads of complement C1q, gC1qR - Functional expression and characterization as a novel vitronectin binding factor. *Journal of Biological Chemistry*, 271, 26739-26744.
- LIU, K., IYODA, T., SATERNUS, M., KIMURA, Y., INABA, K. & STEINMAN, R. M. 2002. Immune tolerance after delivery of dying cells to dendritic cells in situ. *Journal of Experimental Medicine*, 196, 1091-1097.

- LONG, Y. F., MENG, F. X., KONDO, N., IWAMOTO, A. & MATSUDA, Z. 2011. Conserved arginine residue in the membrane-spanning domain of HIV-1 gp41 is required for efficient membrane fusion. *Protein & Cell*, 2, 369-376.
- LOOS, M., HEINZ, H. P., RESKE, K., DLUGONSKA, A. & RUDE, E. 1983. Expression of Endogenous C1q on the Surface of Macrophages. *Immunobiology*, 164, 272-272.
- LOZACH, P. Y., LORTAT-JACOB, H., DE LAVALETTE, A. D., STAROPOLI, I., FOUNG, S., AMARA, A., HOULES, C., FIESCHI, F., SCHWARTZ, O., VIRELIZIER, J. L., ARENZANA-SEISDEDOS, F. & ALTMAYER, R. 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *Journal of Biological Chemistry*, 278, 20358-20366.
- LU, L., ZHU, Y., HUANG, J. H., CHEN, X., YANG, H. W., JIANG, S. B. & CHEN, Y. H. 2008. Surface exposure of the HIV-1 Env cytoplasmic tail LLP2 domain during the membrane fusion process - Interaction with gp41 fusion core. *Journal of Biological Chemistry*, 283, 16723-16731.
- LU, P. D., GALANAKIS, D. K., GHEBREHIWET, B. & PEERSCHKE, E. I. B. 1999. The receptor for the globular "heads" of C1q, gC1q-R, binds to fibrinogen/fibrin and impairs its polymerization. *Clinical Immunology*, 90, 360-367.
- LUO, Y., YU, H. F. & PETERLIN, B. M. 1994. Cellular Protein Modulates Effects of Human-Immunodeficiency-Virus Type-1 Rev. *Journal of Virology*, 68, 3850-3856.
- LYNCH, N. J., REID, K. B. M., VANDENBERG, R. H., DAHA, M. R., LEIGH, L. A. E., GHEBREHIWET, B., LIM, W. B. L. & SCHWAEBLE, W. J. 1997. Characterisation of the rat and mouse homologues of gClqBP, a 33 kDa glycoprotein that binds to the globular 'heads' of Clq. *Febs Letters*, 418, 111-114.
- LYNCH, N. J., REID, K. B. M., VANDENBERG, R. H., DAHA, M. R., LEIGH, L. A. E., GHEBREHIWET, B., LIM, W. B. L. & SCHWAEBLE, W. J. 1997. Characterisation of the rat and mouse homologues of gClqBP, a 33 kDa glycoprotein that binds to the globular 'heads' of Clq. *Febs Letters*, 418, 111-114.
- MAEDA, N., NIGOU, J., HERRMANN, J. L., JACKSON, M., AMARA, A., LAGRANGE, P. H., PUZO, G., GICQUEL, B. & NEYROLLES, O. 2003. The cell surface receptor DC-SIGN discriminates between Mycobacterium species through selective recognition of the mannose caps on lipoarabinomannan. *Journal of Biological Chemistry*, 278, 5513-5516.
- MAHDI, F., MADAR, Z. S., FIGUEROA, C. D. & SCHMALER, A. H. 2002. Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes. *Blood*, 99, 3585-3596.
- MAHDI, F., SHARIAT-MADAR, Z., TODD, R. F., FIGUEROA, C. D. & SCHMAIER, A. H. 2001. Expression and colocalization of cytokeratin 1 and urokinase plasminogen activator receptor on endothelial cells. *Blood*, 97, 2342-2350.
- MALHOTRA, R., HAURUM, J. S., THIEL, S. & SIM, R. B. 1994. Binding of Human Collectins (Sp-a and Mbp) to Influenza-Virus. *Biochemical Journal*, 304, 455-461.
- MALHOTRA, R., WILLIS, A. C., JENSENIUS, J. C., JACKSON, J. & SIM, R. B. 1993. Structure and Homology of Human C1q Receptor (Collectin Receptor). *Immunology*, 78, 341-348.
- MANDERSON, A. P., BOTTO, M. & WALPORT, M. J. 2004. The role of complement in the development of systemic lupus erythematosus. *Annual Review of Immunology*, 22, 431-456.
- MARCEAU, F. & REGOLI, D. 2004. Bradykinin receptor ligands: Therapeutic perspectives. *Nature Reviews Drug Discovery*, 3, 845-852.

- MARQUES, G., ANTON, L. C., BARRIO, E., SANCHEZ, A., RUIZ, S., GAVILANES, F. & VIVANCO, F. 1993. Arginine Residues of the Globular Regions of Human C1q Involved in the Interaction with Immunoglobulin-G. *Journal of Biological Chemistry*, 268, 10393-10402.
- MARTINEZ, O., BRACKENRIDGE, S., EL-IDRISSI, M. E. & PRABHAKAR, B. S. 2005. DC-SIGN, but not sDC-SIGN, can modulate IL-2 production from PMA- and anti-CD3-stimulated primary human CD4 T cells. *International Immunology*, 17, 769-778.
- MASSO, M. 2003. DC-SIGN points the way to a novel mechanism for HIV-1 transmission. *MedGenMed*, 5, 2.
- MATSUDA, K. & YUZAKI, M. 2011. Cbln family proteins promote synapse formation by regulating distinct neurexin signaling pathways in various brain regions. *European Journal of Neuroscience*, 33, 1447-1461.
- MATSUSHITA, M. & FUJITA, T. 1992. Activation of the Classical Complement Pathway by Mannose-Binding Protein in Association with a Novel C1s-Like Serine Protease. *Journal of Experimental Medicine*, 176, 1497-1502.
- MATTHEWS, D. A. & RUSSELL, W. C. 1998. Adenovirus core protein V interacts with p32 - a protein which is associated with both the mitochondria and the nucleus. *Journal of General Virology*, 79, 1677-1685.
- MCEACHERN, A. E., SHELTON, E. R., BHAKTA, S., OBERNOLTE, R., BACH, C., ZUPPAN, P., FUJISAKI, J., ALDRICH, R. W. & JARNAGIN, K. 1991. Expression Cloning of a Rat B2-Bradykinin Receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 7724-7728.
- MCGETTRICK, A. F. & O'NEILL, L. A. J. 2007. Toll-like receptors: key activators of leucocytes and regulator of haematopoiesis. *British Journal of Haematology*, 139, 185-193.
- MCGREAL, E. & GASQUE, P. 2002. Structure-function studies of the receptors for complement C1q. *Biochemical Society Transactions*, 30, 1010-1014.
- MCLACHLAN, A. D. & STEWART, M. 1975. Tropomyosin Coiled-Coil Interactions - Evidence for an Unstaggered Structure. *Journal of Molecular Biology*, 98, 293-304.
- MEDOF, M. E., IIDA, K., MOLD, C. & NUSSENZWEIG, V. 1982. Unique Role of the Complement Receptor Cr-1 in the Degradation of C3b Associated with Immune-Complexes. *Journal of Experimental Medicine*, 156, 1739-1754.
- MEGYERI, M., HARMAT, V., MAJOR, B., VEGH, A., BALCZER, J., HEJA, D., SZILAGYI, K., DATZ, D., PAL, G., ZAVODSZKY, P., GAL, P. & DOBO, J. 2013. Quantitative Characterization of the Activation Steps of Mannan-binding Lectin (MBL)-associated Serine Proteases (MASPs) Points to the Central Role of MASP-1 in the Initiation of the Complement Lectin Pathway. *Journal of Biological Chemistry*, 288, 8922-8934.
- MEI, J., CHEN, B., YUE, H. M. & GUI, J. F. 2008. Identification of a C1q family member associated with cortical granules and follicular cell apoptosis in *Carassius auratus gibelio*. *Molecular and Cellular Endocrinology*, 289, 67-76.
- MELIKYAN, G. B. 2008. Common principles and intermediates of viral protein-mediated fusion: the HIV-1 paradigm. *Retrovirology*, 5.
- MENKE, J. G., BORKOWSKI, J. A., BIERILO, K. K., MACNEIL, T., DERRICK, A. W., SCHNECK, K. A., RANSOM, R. W., STRADER, C. D., LINEMEYER, D. L. & HESS, J. F. 1994. Expression Cloning of a Human B-1 Bradykinin Receptor. *Journal of Biological Chemistry*, 269, 21583-21586.
- MILLER, M. A., CLOYD, M. W., LIEBMANN, J., RINALDO, C. R., ISLAM, K. R., WANG, S. Z. S., MIETZNER, T. A. & MONTELARO, R. C. 1993. Alterations in Cell-Membrane

- Permeability by the Lentivirus Lytic Peptide (Llp-1) of Hiv-1 Transmembrane Protein. *Virology*, 196, 89-100.
- MILLER, M. D., GELEZIUNAS, R., BIANCHI, E., LENNARD, S., HRIN, R., ZHANG, H. C., LU, M. Q., AN, Z. Q., INGALLINELLA, P., FINOTTO, M., MATTU, M., FINNEFROCK, A. C., BRAMHILL, D., COOK, J., ECKERT, D. M., HAMPTON, R., PATEL, M., JARANTOW, S., JOYCE, J., CILIBERTO, G., CORTESE, R., LU, P., STROHL, W., SCHLEIF, W., MCELHAUGH, M., LANE, S., LLOYD, C., LOWE, D., OSBOURN, J., VAUGHAN, T., EMINI, E., BARBATO, G., KIM, P. S., HAZUDA, D. J., SHIVER, J. W. & PESSI, A. 2005. A human monoclonal antibody neutralizes diverse HIV-1 isolates by binding a critical gp41 epitope. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 14759-14764.
- MIR, A., PORTEU, F., LEVY, M., LESAVRE, P. & HALBWACHSMECARELLI, L. 1988. C3b Receptor (Cr-1) on Phagocytic-Cells from Sle Patients - Analysis of the Defect and Familial Study. *Clinical and Experimental Immunology*, 73, 461-466.
- MITCHELL, D. A., FADDEN, A. J. & DRICKAMER, K. 2001. A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR - Subunit organization and binding to multivalent ligands. *Journal of Biological Chemistry*, 276, 28939-28945.
- MITCHELL, D. A., FADDEN, A. J. & DRICKAMER, K. 2001. A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR - Subunit organization and binding to multivalent ligands. *Journal of Biological Chemistry*, 276, 28939-28945.
- MOHAN, K. V. K., GHEBREHIWET, B. & ATREYA, C. D. 2002. The N-terminal conserved domain of rubella virus capsid interacts with the C-terminal region of cellular p32 and overexpression of p32 enhances the viral infectivity. *Virus Research*, 85, 151-161.
- MUNOZ-BARROSO, I., SALZWEDEL, K., HUNTER, E. & BLUMENTHAL, R. 1999. Role of the membrane-proximal domain in the initial stages of human immunodeficiency virus type 1 envelope glycoprotein-mediated membrane fusion (vol 73, pg 6089, 1999). *Journal of Virology*, 73, 9693-9693.
- NAUTA, A. J., BOTTAZZI, B., MANTOVANI, A., SALVATORI, G., KISHORE, U., SCHWAEBLE, W. J., GINGRAS, A. R., TZIMA, S., VIVANCO, F., EGIDO, J., TIJSMA, O., HACK, E. C., DAHA, M. R. & ROOS, A. 2003. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *European Journal of Immunology*, 33, 465-473.
- NAVRATIL, J. S., WATKINS, S. C., WISNIESKI, J. J. & AHEARN, J. M. 2001. The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. *Journal of Immunology*, 166, 3231-9.
- NAYAK, A., FERLUGA, J., TSOLAKI, A. G. & KISHORE, U. 2010. The non-classical functions of the classical complement pathway recognition subcomponent C1q. *Immunology Letters*, 131, 139-150.
- NEPOMUCENO, R. R., HENSCHENEDMAN, A. H., BURGESS, W. H. & TENNER, A. J. 1997. cDNA cloning and primary structure analysis of C1qR(p), the human C1q/MBL/SPA receptor that mediates enhanced phagocytosis in vitro. *Immunity*, 6, 119-129.
- NEPOMUCENO, R. R., RUIZ, S., PARK, M. & TENNER, A. J. 1999. C1qR(P) is a heavily O-glycosylated cell surface protein involved in the regulation of phagocytic activity. *Journal of Immunology*, 162, 3583-3589.

- NGUYEN, T., GHEBREHIWET, B. & PEERSCHKE, E. I. B. 2000. Staphylococcus aureus protein A recognizes platelet gC1qR/p33: a novel mechanism for staphylococcal interactions with platelets. *Infection and Immunity*, 68, 2061-2068.
- NI, K. & ONEILL, H. C. 1997. The role of dendritic cells in T cell activation. *Immunology and Cell Biology*, 75, 223-230.
- NORSWORTHY, P. J., FOSSATI-JIMACK, L., CORTES-HERNANDEZ, J., TAYLOR, P. R., BYGRAVE, A. E., THOMPSON, R. D., NOURSHARGH, S., WALPORT, M. J. & BOTTO, M. 2004. Murine CD93 (C1qRp) contributes to the removal of apoptotic cells in vivo but is not required for C1q-mediated enhancement of phagocytosis. *Journal of Immunology*, 172, 3406-3414.
- OGDEN, C. A., DECATHEHNEAU, A., HOFFMANN, P. R., BRATTON, D., GHEBREHIWET, B., FADOK, V. A. & HENSON, P. M. 2001. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *Journal of Experimental Medicine*, 194, 781-795.
- OIKI, S. & OKADA, Y. 1988. C1q Induces Chemotaxis and K⁺ Conductance Activation Coupled to Increased Cytosolic Ca²⁺ in Mouse Fibroblasts. *Journal of Immunology*, 141, 3177-3185.
- PAIDASSI, H., TACNET-DELORME, P., GARLATTI, V., DARNAULT, C., GHEBREHIWET, B., GABORIAUD, C., ARLAUD, G. J. & FRACHET, P. 2008. C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. *Journal of Immunology*, 180, 2329-2338.
- PAIDASSI, H., TACNET-DELORME, P., VERNERET, M., GABORIAUD, C., HOUEN, G., DUUS, K., LING, W. L., ARLAUD, G. J. & FRACHET, P. 2011. Investigations on the C1q-Calreticulin-Phosphatidylserine Interactions Yield New Insights into Apoptotic Cell Recognition. *Journal of Molecular Biology*, 408, 277-290.
- PANCERA, M., MAJEED, S., BAN, Y., CHEN, L., HUANG, C., KONG, L., KWON, Y., STUCKEY, J., ZHOU, T., ROBINSON, J., SCHIEF, W., SODROSKI, J., WYATT, R. & KWONG, P. 2009. Structure of HIV-1 gp41 interactive region: layered architecture and basis of conformational mobility. *Retrovirology*, 6.
- PASARE, C. & MEDZHITOV, R. 2003. Toll pathway-dependent blockade of CD4(+)CD25(+) T cell-mediated suppression by dendritic cells. *Science*, 299, 1033-1036.
- PEERSCHKE, E. I. B. & GHEBREHIWET, B. 1997a. C1q augments platelet activation by aggregated immunoglobulins. *Thrombosis and Haemostasis*, P1967-P1967.
- PEERSCHKE, E. I. B. & GHEBREHIWET, B. 1997b. C1q augments platelet activation in response to aggregated Ig. *Journal of Immunology*, 159, 5594-5598.
- PEERSCHKE, E. I. B., REID, K. B. M. & GHEBREHIWET, B. 1994. Identification of a Novel 33-Kda C1q-Binding Site on Human Blood-Platelets. *Journal of Immunology*, 152, 5896-5901.
- PEERSCHKE, E. I. B., YIN, W., GRIGG, S. E. & GHEBREHIWET, B. 2006. Blood platelets activate the classical pathway of human complement. *Journal of Thrombosis and Haemostasis*, 4, 2035-2042.
- PEERSCHKE, E. I. B., YIN, W., GRIGG, S. E. & GHEBREHIWET, B. 2006. Blood platelets activate the classical pathway of human complement. *Journal of Thrombosis and Haemostasis*, 4, 2035-2042.
- PEERSCHKE, E. I., MURPHY, T. K. & GHEBREHIWET, B. 2001. The gC1qR/p33 is constitutively expressed on the surface membrane of human platelets. *Blood*, 98, 516a-516a.

- PETERSON, K. L., ZHANG, W. B., LU, P. D., KEILBAUGH, S. A., PEERSCHKE, E. I. B. & GHEBREHIWET, B. 1997. The C1q-binding cell membrane proteins cC1q-R and gC1q-R are released from activated cells: Subcellular distribution and immunochemical characterization. *Clinical Immunology and Immunopathology*, 84, 17-26.
- PETRY, F., REID, K. B. M. & LOOS, M. 1991. Gene-Expression of the α -Chain and B-Chain of Mouse C1q in Different Tissues and the Characterization of the Recombinant α -Chain. *Journal of Immunology*, 147, 3988-3993.
- PISALYAPUT, K. & TENNER, A. J. 2008. Complement component C1q inhibits beta-amyloid- and serum amyloid P-induced neurotoxicity via caspase- and calpain-independent mechanisms. *Journal of Neurochemistry*, 104, 696-707.
- PIXLEY, R. A., ESPINOLA, R. G., GHEBREHIWET, B., JOSEPH, K., KAO, A., BDEIR, K., CINES, D. B. & COLMAN, R. W. 2011. Interaction of high-molecular-weight kininogen with endothelial cell binding proteins suPAR, gC1qR and cytokeratin 1 determined by Surface Plasmon Resonance (BiaCore). *Thrombosis and Haemostasis*, 105, 1053-1059.
- POLTORAK, A., HE, X. L., SMIRNOVA, I., LIU, M. Y., VAN HUFFEL, C., DU, X., BIRDWELL, D., ALEJOS, E., SILVA, M., GALANOS, C., FREUDENBERG, M., RICCIARDI-CASTAGNOLI, P., LAYTON, B. & BEUTLER, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science*, 282, 2085-2088.
- PPELMELK, B. J., VAN DIE, I., VAN VLIET, S. J., VANDENBROUCKE-GRAULS, C. M. J. E., GEIJTENBEEK, T. B. H. & VAN KOOYK, Y. 2003. Cutting edge: Carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *Journal of Immunology*, 170, 1635-1639.
- POHLMANN, S., ZHANG, J., BARIBAUD, F., CHEN, Z. W., LESLIE, G., LIN, G., GRANELLI-PIPERNO, A., DOM, R. W., RICE, C. M. & MCSKEATING, J. A. 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *Journal of Virology*, 77, 4070-4080.
- PRABAGAR, M. G., DO, Y., RYU, S., PARK, J. Y., CHOI, H. J., CHOI, W. S., YUN, T. J., MOON, J., CHOI, I. S., KO, K., SHIN, C. Y., CHEONG, C. & KANG, Y. S. 2013. SIGN-R1, a C-type lectin, enhances apoptotic cell clearance through the complement deposition pathway by interacting with C1q in the spleen. *Cell Death and Differentiation*, 20, 535-545.
- QUINKAL, I., HERNANDEZ, J. F., CHEVALLIER, S., ARLAUD, G. J. & VERNET, T. 1999. Mapping of the interaction between the immunodominant loop of the ectodomain of HIV-1 gp41 and human complement protein C1q. *European Journal of Biochemistry*, 265, 656-663.
- RANDAZZO, B. P., FLEIT, H. B., KAPLAN, A. P. & GHEBREHIWET, B. 1988. Expression of Functional Cell-Surface C1-Inactivator by U937 Cells. *Clinical Immunology and Immunopathology*, 49, 463-477.
- REGOLI, D. & BARABE, J. 1980. Pharmacology of Bradykinin and Related Kinins. *Pharmacological Reviews*, 32, 1-46.
- REISINGER EC, VOGETSEDER, W, BERZOW,D, KOFLER, D, BITTERLICH G, LEHR, HA, WACHTER,H DIERICH, MP. 1990. Complement-mediated enhancement of HIV-1 infection of the monoblastoid cell line U937. *AIDS*, 961-5
- RONNBLOM, L. & ALM, G. V. 2001. A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus. *Journal of Experimental Medicine*, 194, F59-F63.

- ROSSI, V., CSEH, S., BALLY, I., THIELENS, N. M., JENSENIUS, J. C. & ARLAUD, G. J. 2001. Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and-2. *Journal of Biological Chemistry*, 276, 40880-40887.
- ROUMENINA, L. T., KANTARDJIEV, A. A., ATANASOV, B. P., WATERS, P., GADJEVA, M., REID, K. B. M., MANTOVANI, A., KISHORE, U. & KOJOUHAROVA, M. S. 2005. Role of Ca²⁺ in the electrostatic stability and the functional activity of the globular domain of human C1q. *Biochemistry*, 44, 14097-14109.
- ROUMENINA, L. T., POPOV, K. T., BUREEVA, S. V., KOJOUHAROVA, M., GADJEVA, M., RABHERU, S., THAKRAR, R., KAPLUN, A. & KISHORE, U. 2008. Interaction of the globular domain of human C1q with *Salmonella typhimurium* lipopolysaccharide. *Biochimica Et Biophysica Acta-Proteins and Proteomics*, 1784, 1271-1276.
- ROUMENINA, L. T., RUSEVA, M. M., ZLATAROVA, A., GHAI, R., KOLEV, M., OLOVA, N., GADJEVA, M., AGRAWAL, A., BOTTAZZI, B., MANTOVANI, A., REID, K. B. M., KISHORE, U. & KOJOUHAROVA, M. S. 2006. Interaction of C1q with IgG1, C-reactive protein and pentraxin 3: Mutational studies using recombinant globular head modules of human C1q A, B, and C chains. *Biochemistry*, 45, 4093-4104.
- ROUSSO, I., MIXON, M. B., CHEN, B. K. & KIM, P. S. 2000. Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 13523-13525.
- ROZANOV, D. V., GHEBREHIWET, B., POSTNOVA, T. I., EICHINGER, A., DERYUGINA, E. I. & STRONGIN, A. Y. 2002. The hemopexin-like C-terminal domain of membrane type 1 matrix metalloproteinase regulates proteolysis of a multifunctional protein, gC1qR. *Journal of Biological Chemistry*, 277, 9318-9325.
- RUIZ, S., HENSCHENEDMAN, A. H. & TENNER, A. J. 1995. Localization of the site on the complement component C1q required for the stimulation of neutrophil superoxide production. *Journal of Biological Chemistry*, 270, 30627-30634.
- SARKAR, R., MITRA, D. & CHAKRABARTI, S. 2013. HIV-1 Gp120 Protein Downregulates Nef Induced IL-6 Release in Immature Dendritic Cells through Interplay of DC-SIGN. *Plos One*, 8.
- SCHERER, P. E., WILLIAMS, S., FOGLIANO, M., BALDINI, G. & LODISH, H. F. 1995. A Novel Serum-Protein Similar to C1q, Produced Exclusively in Adipocytes. *Journal of Biological Chemistry*, 270, 26746-26749.
- SCHNARE, M., BARTON, G. M., HOLT, A. C., TAKEDA, K., AKIRA, S. & MEDZHITOV, R. 2001. Toll-like receptors control activation of adaptive immune responses. *Nature Immunology*, 2, 947-950.
- SCHULTZ, K. T. & GRIEDER, F. 1987. Structure and Function of the Immune-System. *Toxicologic Pathology*, 15, 262-264.
- SCHUR, P. H. 1982. Complement and Lupus-Erythematosus. *Arthritis and Rheumatism*, 25, 793-798.
- SELLAR, G. C., BLAKE, D. J. & REID, K. B. M. 1991. Characterization and Organization of the Genes Encoding the α -Chains, B-Chains and C-Chains of Human-Complement Subcomponent C1q - the Complete Derived Amino-Acid-Sequence of Human C1q. *Biochemical Journal*, 274, 481-490.
- SETHI, S., HERRMANN, M., ROLLER, J., VON MULLER, L., PEERSCHKE, E. I., GHEBREHIWET, B., BAJRIC, I., MENGER, M. D. & LASCHKE, M. W. 2011. Blockade of gC1qR/p33, a receptor for C1q, inhibits adherence of *Staphylococcus aureus* to the microvascular endothelium. *Microvascular Research*, 82, 66-72.

- SHANG, L., YUE, L. & HUNTER, E. 2008. Role of the membrane-spanning domain of human immunodeficiency virus type 1 envelope glycoprotein in cell-cell fusion and virus infection. *Journal of Virology*, 82, 5417-5428.
- SHAPIRO, L. & SCHERER, P. E. 1998. The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Current Biology*, 8, 335-338.
- SHIMONO, C., MANABE, R., YAMADA, T., FUKUDA, S., KAWAI, J., FURUTANI, Y., TSUTSUI, K., IKENAKA, K., HAYASHIZAKI, Y. & SEKIGUCHI, K. 2010. Identification and characterization of nCLP2, a novel C1q family protein expressed in the central nervous system. *Journal of Biochemistry*, 147, 565-579.
- SIM, R. B., KISHORE, U., VILLIERS, C. L., MARCHE, P. N. & MITCHELL, D. A. 2007. C1q binding and complement activation by prions and amyloids. *Immunobiology*, 212, 355-362.
- SINGH, S. K., STEPHANI, J., SCHAEFER, M., KALAY, H., GARCIA-VALLEJO, J. J., DEN HAAN, J., SAELAND, E., SPARWASSER, T. & VAN KOOYK, Y. 2009. Targeting glycan modified OVA to murine DC-SIGN transgenic dendritic cells enhances MHC class I and II presentation. *Molecular Immunology*, 47, 164-174.
- SOILLEUX, E. J., BARTEN, R. & TROWSDALE, J. 2000. Cutting edge: DC-SIGN; a related gene, DC-SIGNR; and CD23 form a cluster on 19p13. *Journal of Immunology*, 165, 2937-2942.
- SOLDER, B. M., SCHULZ, T. F., HENGSTER, P., LOWER, J., LARCHER, C., BITTERLICH, G., KURTH, R., WACHTER, H. & DIERICH, M. P. 1989. Hiv and Hiv-Infected Cells Differentially Activate the Human-Complement System Independent of Antibody. *Immunology Letters*, 22, 135-145.
- SRIVASTAVA, P. 2002. Interaction of heat shock proteins with peptides and antigen presenting cells: Chaperoning of the innate and adaptive immune responses. *Annual Review of Immunology*, 20, 395-425.
- STEGMANN, T., DELFINO, J. M., RICHARDS, F. M. & HELENIUS, A. 1991. The Ha2 Subunit of Influenza Hemagglutinin Inserts into the Target Membrane Prior to Fusion. *Journal of Biological Chemistry*, 266, 18404-18410.
- STEINBERGER, P., SZEKERES, A., WILLE, S., STOCKL, J., SELENKO, N., PRAGER, E., STAFFLER, G., MADIC, O., STOCKINGER, H. & KNAPP, W. 2002. Identification of human CD93 as the phagocytic C1q receptor (C1qRp) by expression cloning. *Journal of Leukocyte Biology*, 71, 133-140.
- STEPHAN, A. H., MADISON, D. V., MATEOS, J. M., FRASER, D., COUTELLIER, L., LOVELETT, E., TSAI, H. H., HUANG, E., ROWITCH, D., KIM, L., TENNER, A., SHAMLOO, M. & BARRES, B. A. 2012. A role for C1q in normal brain aging. *Immunobiology*, 217, 1133-1133.
- STOCKER, H., SCHELLER, C. & JASSOY, C. 2000. Destruction of primary CD4(+) T cells by cell-cell interaction in human immunodeficiency virus type 1 infection in vitro. *Journal of General Virology*, 81, 1907-1911.
- STOIBER, H., EBENBICHLER, C. F., THIELENS, N. M., ARLAUD, G. J. & DIERICH, M. P. 1995. Hiv-1 Rsgp41 Depends on Calcium for Binding of Human C1q but Not for Binding of Gp120. *Molecular Immunology*, 32, 371-374.
- SZABO, J., CERVENAK, L., TOTH, F. D., PROHASZKA, Z., HORVATH, L., KEREEKES, K., BECK, Z., BACSI, A., ERDEI, A., PEERSCHKE, E. I. B., FUST, G. & GHEBREHIWET, B. 2001. Soluble gC1q-R/p33, a cell protein that binds to the globular "heads" of C1q, effectively inhibits the growth of HIV-1 strains in cell cultures. *Clinical Immunology*, 99, 222-231.
- TACNET-DELORME, P., CHEVALLIER, S. & ARLAND, G. J. 2001. beta-amyloid fibrils activate the C1 complex of complement under physiological conditions: Evidence for a

- binding site for A beta on the C1q globular regions. *Journal of Immunology*, 167, 6374-6381.
- TAKEDA, K., KAISHO, T. & AKIRA, S. 2003. Toll-like receptors. *Annual Review of Immunology*, 21, 335-376.
- TAKEUCHI, O., HOSHINO, K., KAWAI, T., SANJO, H., TAKADA, H., OGAWA, T., TAKEDA, K. & AKIRA, S. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*, 11, 443-451.
- TANG, Y. T., HU, T. H., ARTERBURN, M., BOYLE, B., BRIGHT, J. M., PALENCIA, S., EMTAGE, P. C. & FUNK, W. D. 2005. The complete complement of C1q-domain-containing proteins in *Homo sapiens*. *Genomics*, 86, 100-111.
- TAS, S. W., KLICKSTEIN, L. B., BARBASHOV, S. F. & NICHOLSON-WELLER, A. 1999. C1q and C4b bind simultaneously to CR1 and additively support erythrocyte adhesion. *Journal of Immunology*, 163, 5056-5063.
- TENNER, A. J. 1998. C1q receptors: Regulating specific functions of phagocytic cells. *Immunobiology*, 199, 250-264.
- THIELENS, N. M., BALLY, I. M., EBENBICHLER, C. F., DIERICH, M. P. & ARLAUD, G. J. 1993. Further Characterization of the Interaction between the C1q Subcomponent of Human C1 and the Transmembrane Envelope Glycoprotein Gp41 of Hiv-1. *Journal of Immunology*, 151, 6583-6592.
- TURVILLE, S. G., ARTHOS, J., MAC DONALD, K., LYNCH, G., NAIF, H., CLARK, G., HART, D. & CUNNINGHAM, A. L. 2001. HIV gp120 receptors on human dendritic cells. *Blood*, 98, 2482-2488.
- VALDES, G., KAUFMANN, P., CORTHORN, J., ERICES, R., BROSNIHAN, K. B. & JOYNER-GRANTHAM, J. 2009. Vasodilator factors in the systemic and local adaptations to pregnancy. *Reproductive Biology and Endocrinology*, 7.
- VAN DEN BERG, R. H., FABER-KROL, M. C., SIM, R. B. & DAHA, M. R. 1998. The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells. *Journal of Immunology*, 161, 6924-6930.
- VAN LEEUWEN, H. C. & O'HARE, P. 2001. Retargeting of the mitochondrial protein p32/gC1qR to a cytoplasmic compartment and the cell surface. *Journal of Cell Science*, 114, 2115-2123.
- VEGH, Z., GOYARTS, E. C., ROZENGARTEN, K., MAZUMDER, A. & GHEBREHIWET, B. 2003. Maturation-dependent expression of C1q binding proteins on the cell surface of human monocyte-derived dendritic cells. *International Immunopharmacology*, 3, 39-51.
- VEGH, Z., KEW, R. R., GRUBER, B. L. & GHEBREHIWET, B. 2006. Chemotaxis of human monocyte-derived dendritic cells to complement component C1q is mediated by the receptors gC1qR and cC1qR. *Molecular Immunology*, 43, 1402-1407.
- VIEILLARD, V., STROMINGER, J. L. & DEBRE, P. 2005. NK cytotoxicity against CD4(+) T cells during HIV-1 infection: A gp41 peptide induces the expression of an NKp44 ligand. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 10981-10986.
- VOLANAKIS, J. E. 1982. Solubilization of C-Reactive Protein (Crp)-Pneumococcal C-Polysaccharide (Pnc) Precipitates by Complement. *Molecular Immunology*, 19, 1408-1408.

- VOLANAKIS, J. E. 2001. Human C-reactive protein: expression, structure, and function. *Molecular Immunology*, 38, 189-197.
- WANG, Y. L., FINAN, J. E., MIDDELDORP, J. M. & HAYWARD, S. D. 1997. P32/TAP, a cellular protein that interacts with EBNA-1 of Epstein-Barr virus. *Virology*, 236, 18-29.
- WARD, J., BONAPARTE, M., SACKS, J., GUTERMAN, J., FOGLI, M., MAVILIO, D. & BARKER, E. 2007. HIV modulates the expression of ligands important in triggering natural killer cell cytotoxic responses on infected primary T-cell blasts. *Blood*, 110, 1207-1214.
- WATTHANASUROROT, A., JIRAVANICHPAISAL, P., SODERHALL, I. & SODERHALL, K. 2010. A gC1qR Prevents White Spot Syndrome Virus Replication in the Freshwater Crayfish *Pacifastacus leniusculus*. *Journal of Virology*, 84, 10844-10851.
- WEIS, W. I., DRICKAMER, K. & HENDRICKSON, W. A. 1992. Structure of a C-Type Mannose-Binding Protein Complexed with an Oligosaccharide. *Nature*, 360, 127-134.
- WEIS, W. I., TAYLOR, M. E. & DRICKAMER, K. 1998. The C-type lectin superfamily in the immune system. *Immunological Reviews*, 163, 19-34.
- WERFEL, T., KIRCHHOFF, K., WITTMANN, M., BEGEMANN, G., KAPP, A., HEIDENREICH, F., GOTZE, O. & ZWIRNER, J. 2000. Activated human T lymphocytes express a functional C3a receptor. *Journal of Immunology*, 165, 6599-6605.
- YANG, P. L., AI, L. S., HUANG, S. C., LI, H. F., CHAN, W. E., CHANG, C. W., KO, C. Y. & CHEN, S. S. L. 2010. The Cytoplasmic Domain of Human Immunodeficiency Virus Type 1 Transmembrane Protein gp41 Harbors Lipid Raft Association Determinants. *Journal of Virology*, 84, 59-75.
- YAO, Z. Q., EISEN-VANDERVELDE, A., WAGGONER, S. N., CALE, E. M. & HAHN, Y. S. 2004. Direct binding of hepatitis C virus core to gC1qR on CD4(+) and CD8(+) T cells leads to impaired activation of Lck and Akt. *Journal of Virology*, 78, 6409-6419.
- YAO, Z. Q., EISEN-VANDERVELDE, A., WAGGONER, S. N., CALE, E. M. & HAHN, Y. S. 2004. Direct binding of hepatitis C virus core to gC1qR on CD4(+) and CD8(+) T cells leads to impaired activation of Lck and Akt. *Journal of Virology*, 78, 6409-6419.
- YIN, W., GHEBREHIWET, B., WEKSLER, B. & PEERSCHKE, E. I. 2007. Classical pathway complement activation on human endothelial cells. *Molecular Immunology*, 44, 2228-2234.
- YOUNG, K. R., AMBRUS, J. L., MALBRAN, A., FAUCI, A. S. & TENNER, A. J. 1991. Complement Subcomponent C1q Stimulates Ig Production by Human Lymphocytes-B. *Journal of Immunology*, 146, 3356-3364.
- YU, L., LOEWENSTEIN, P. M., ZHANG, Z. H. & GREEN, M. 1995. In-Vitro Interaction of the Human-Immunodeficiency-Virus Type-1 Tat Transactivator and the General Transcription Factor Tfiib with the Cellular Protein Tap. *Journal of Virology*, 69, 3017-3023.
- ZHAO, N., PANG, B., SHYU, C. R. & KORKIN, D. 2011. Charged residues at protein interaction interfaces: Unexpected conservation and orchestrated divergence. *Protein Science*, 20, 1275-1284.
- ZUTTER, M. M. & EDELSON, B. T. 2003. Mast cell-mediated inflammatory responses require the alpha 2 beta 1 integrin. *Blood*, 102, 524a-524a.
- ZUTTER, M. M. & EDELSON, B. T. 2007. The alpha 2 beta 1 integrin: A novel collectin/C1q receptor. *Immunobiology*, 212, 343-353.