INVESTIGATION OF C1Q REGULATION OF MONOCYTE DEVELOPMENT

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Dennis Teo Boon Heng

20 August 2013

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Summary

C1q plays an important role in innate immunity. By acting as the recognition subunit in the classical pathway, C1q helps in the removal of invading pathogens by activating the complement system. Other than its involvement in the complement system, C1q is also involved in many functions such as clearance of apoptotic cells, modulation of immune cells and various cellular processes. Thus, C1q is essential in health but is also implicated in the pathogenesis of several diseases such as Alzheimer's disease, atherosclerosis and rheumatoid arthritis. Furthermore, hereditary or acquired C1q deficiency is associated with systemic lupus erythematosus (SLE).

Despite being an abundant serum protein, serum C1q may not be involved in regulating the functions of various cell types as it may have limited access to tissues. Thus, local synthesis of C1q is crucial in mediating its diverse functions. In view of the importance of local C1q biosynthesis, we investigated possible cellular sources of C1q. Osteoclasts were chosen based on the similarities that the cells have with macrophages and dendritic cells (DCs), known C1q producers in the body.

Osteoclasts were first differentiated from isolated human monocytes and the phenotypes were characterized. Using multiple techniques, C1q was consistently detected in the cultured osteoclasts at both the mRNA and protein levels. In cultured osteoclasts, mononucleated osteoclasts or preosteoclasts preferentially produced C1q, suggesting a role for C1q in osteoclast differentiation. Furthermore, C1q was shown to be present in endogenous preosteoclasts and mature osteoclasts of human femoral bone samples. C1q production in cultured osteoclasts can be regulated by cytokines and Toll-like receptor (TLR) ligands. IFNγ induced a large increase in the production of C1q by both preosteoclasts and mature osteoclasts. The role of C1q in the bone microenvironment is investigated and immobilized, but not soluble, C1q was shown to augment osteoclastogenesis.

Existing data and our study show that C1q regulates the development of monocytes. In addition, C1q affects the morphologies of cells which may be linked to its effects on monocyte development. Thus, we proceed to determine the C1q receptor involved in mediating C1q-induced morphological changes.

Immobilized C1q caused cells to be more rounded and led to the formation of cell clusters. The effects were not only applicable to monocytes but all cell types used in the study were affected to varying extent. The morphological changes were accompanied by decreased adherence of the cells and a correlation was observed in which cells showing more changes in cellular morphology had decreasing adherence. However, such effects were not observed in cells incubated with soluble C1q which corresponded to the results obtained for osteoclast differentiated in the presence of soluble C1q. Known C1q receptors such as $\alpha_2\beta_1$, CD35, CD91, CD93 and gC1qR were not responsible for mediating the effects of immobilized C1q as determined by flow cytometry and antibody blocking experiments. Interestingly, commercial anti-C1q antibodies enhanced the kinetics of immobilized C1q though anti-C1q autoantibodies of SLE patients did not exert any effects on the kinetics of immobilized C1q.

(488 words)

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List of abbreviations

ABC	Avidin-biotin complex
αMEM	Alpha minimum essential medium
AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammonium persulfate
BCS	Bovine calf serum
BSA	Bovine serum albumin
C1q	Complement component 1, subcomponent q
C1qRp	C1q receptor for phagocytosis
CAII	Carbonic anhydrase II
CatK	Cathepsin K
CD	Cluster of differentiation
cDNA	Complementary DNA
CHX	Cycloheximide
CLR	C-type lectin receptor
CR1	Complement receptor 1
CRP	C-reactive protein
CTR	Calcitonin receptor
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEC	Decidual endothelial cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
F-actin	Filamentous actin
FAK	Focal adhesion kinase
Fc	Fragment crytallizable
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Fz	Frizzled
GM-CSF	Granulocyte macrophage-colony stimulating factor
HI	Heat inactivated
HRP	Horse radish peroxidase
ICAM	Intercellular cell adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
kD	Kilodalton
KIR	Killer cell immunoglobulin-like receptor
LAIR	Leukocyte-associated Ig-like receptor
LPS	Lipopolysaccharide

LRP	Low density lipoprotein-related protein
LTA	Lipoteichoic acid
LHR	Long homologous repeat
MAC	Membrane attack complex
M-CSF	Macrophage-colony stimulating factor
M-CSFR	M-CSF receptor
MHC	Major histocompatibility class
mRNA	Messenger RNA
NFATc1	Nuclear factor of activated T cells cytoplasmic 1
NF-κB	Nuclear factor kappa-B
NK	Natural kill
OD	Optical density
PAMP	Pattern associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PE	Phycoerythrin
PFA	Paraformaldehyde
PGN	Peptidoglycan
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real-time PCR
RAGE	Receptor for advanced glycation endproducts
RANK	Receptor activator of NF-κB
RANKL	RANK ligand
RIG	Retinoic acid-inducible gene
RLR	Rig-I-like receptor
RNA	Ribonucleic acid
RPMI	RPMI-1640 culture medium
RQ	Relative quantification
SAP	Serum amyloid protein
SCR	Short consensus repeat
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SP	Surfactant protein
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF	Tumor necrosis factor
TPBS	Tris-PBS
TRAP	Tartrate-resistant acid phosphatase
Tris	Tri-hydroxymethyl-aminomethane
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

List of publication

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Chapter 1 – Introduction

1.1 The immune system

The immune system is traditionally divided into two branches namely the innate and adaptive immunity (Dempsey *et al.*, 2003; Medzhitov and Janeway, 2000). Innate immunity exists much earlier than adaptive immunity and can be found in almost all muticellular organisms. By contrast, adaptive immunity is only present in vertebrates and cartilaginous fish.

The main difference between these two immune systems is the effector mechanism deployed for immune recognition. Innate immunity depends mainly on germline-encoded receptors which recognize highly conversed molecular patterns of pathogens (Dempsey *et al.*, 2003; Medzhitov and Janeway, 2000). On the other hand, adaptive immunity displays more elasticity in which the receptors are generated somatically. This gives rise to a huge repertoire of receptors that are able to recognize unique antigens of the pathogens (Dempsey *et al.*, 2003; Medzhitov and Janeway, 2000). For the immune system to be effective, the interplay and regulation of these 2 branches of immune systems are highly important.

1.1.1 Adaptive immunity

Adaptive immunity is able to clear infections more effectively than innate immunity. It depends mainly on the lymphocytes – the T and B cells – which have a vast repertoire of receptors that can recognize different antigens. Furthermore, adaptive immunity provides immunological memory and can deal with subsequent infections faster and more efficiently (Litman *et al.*, 2010).

1.1.1.1 Antigen presenting cells (APCs)

Providing the link between innate and adaptive immunity, APCs are critical regulators of the immune system (Vyas *et al.*, 2008). Present in many tissues, they carry out a sentinel function in detecting pathogens. Upon encountering pathogens, these cells internalize and process these pathogens. They then present the proteolytic peptides in association with major histocompatibility complex (MHC) and engage antigen-specific T cells, resulting in the activation of the adaptive immunity. Depending on the MHC molecule – MHC class I or MHC class II molecule – cell-mediated or humoral immunity will be induced respectively.

DCs, macrophages and B cells are professional APCs and they possess several characteristics which allow efficient antigen presentation (Guermonprez *et al.*, 2002; Rodriguez-Pinto, 2005; Unanue, 1984). Recent studies have also indicated that osteoclasts are able to present antigens to T cells and activate adaptive immunity (Grassi *et al.*, 2011; Kiesel *et al.*, 2009; Li *et al.*, 2010).

1.1.1.2 T cells

Two major classes exist in T cells and these two classes differ in their effector functions (Koch and Radtke, 2011). They are identified by the expression of cell surface proteins CD4 and CD8. CD4 T cells are T helper cells which are involved in the activation of humoral immunity through the recognition of MHC class II-peptide complex presented by APCs (Vyas *et al.*, 2008). On the other hand, CD8 T cells are cytotoxic T cells which recognize MHC class I-peptide complex of APC or host cells and are involved in cell-mediated immunity (Vyas *et al.*, 2008).

During development in the thymus, T cells undergo somatic rearrangement of the genes encoding T cell receptor (TCR) (Koch and Radtke, 2011; Litman *et al.*, 2010). This gives rise to vast numbers of T cell clones

having unique TCRs which recognize different peptides. Positive and negative selection allows T cells which recognize self MHC molecules but not self peptide complexed with self MHC molecule to survive (Koch and Radtke, 2011). This ensures that the selected T cells are MHC-restricted and self-tolerant.

1.1.1.3 B cells

Similar to the T cells, B cells also undergo somatic rearrangement for the genes encoding B cell receptor (BCR – membrane bound antibody) in the bone marrow (Hardy and Hayakawa, 2001; LeBien and Tedder, 2008; Litman *et al.*, 2010). This gives rise to a high repertoire of B cell clones which are able to recognize diverse antigens. To prevent autoreactive B cells, receptor editing is triggered for autoreactive BCRs before clonal deletion is activated. Clonal deletion removes B cells that have high affinity to self antigen ensuring self tolerance (Hardy and Hayakawa, 2001; LeBien and Tedder, 2008).

Upon activation by armed T helper cells, B cells differentiate into plasma cells and this enables the secretion of antibodies (LeBien and Tedder, 2008). Differentiation of B cells is accompanied by somatic hypermutation and class switching which increases the repertoire for effective immune response.

Despite the diversity and versatility of adaptive immunity, 4 -7 days are required for antigen presentation and clonal expansion so that the adaptive immune system can mount an effective immune response (Dempsey *et al.*, 2003). During this period, pathogens are able to damage the host. Thus, innate immunity plays an important role in controlling the pathogens as its response is immediate. This provides sufficient time for the adaptive immune response to mature and assist in the eventual clearance of invading pathogens if the innate immune response is insufficient in removing the pathogens.

1.1.2 Innate immunity

Besides the physical and chemical barriers that prevent the entry of pathogens, innate immunity is the first line of defence against these pathogens. The innate immune system consists of cellular and humoral responses.

1.1.2.1 Cellular response

Upon encountering pathogens, cellular mediators such as macrophages, neutrophils and natural killer (NK) cells are activated and recruited to the site of infection (Soehnlein and Lindbom, 2010). Macrophages and neutrophils are phagocytes which are responsible for the phagocytosis of the microbes. Phagocytes depend on germline receptors that recognize conserved motifs on pathogens but not on host cells (Dempsey *et al.*, 2003). These phagocytic receptors include scavenger receptors, mannose receptor and Dectin-1 which bind to surface motifs of pathogens and mediate their uptake (Underhill and Ozinsky, 2002). In addition, pathogens are opsonized by humoral mediators such as complement and antibodies and this allows the phagocytosis of pathogens by complement receptors and Fc receptors respectively (Underhill and Ozinsky, 2002). After ingesting the pathogens, the phagocytes produce toxic mediators such as nitric oxide, reactive oxygen species and hydrogen peroxide to kill the pathogens.

NK cells are involved mainly in anti-viral immunity. They express various activating and inhibitory receptors that help to stimulate or dampen cell reactivity respectively (Bryceson *et al.*, 2006; Vivier *et al.*, 2011). An example is the MHC class I-specific inhibitory receptors that include killer cell immunoglobulin-like receptors (KIRs) and CD94/NKG2A heterodimers (Bryceson *et al.*, 2006; Vivier *et al.*, 2011). In virus-infected cells, cell surface MHC class I molecules are downregulated and the lack of signaling from inhibitory receptors activate the NK cells, resulting in the killing of the virus-infected cells (Vivier *et al.*, 2011).

1.1.2.2 Humoral response

Other than cellular response of innate immunity, the humoral response also plays an important role in the first line of defence against invading pathogens. Anti-microbial peptides, cytokines, chemokines and complement form this part of innate immunity (Dempsey *et al.*, 2003).

Anti-microbial peptides are small, cationic proteins and an example is defensins. These peptides interact with negatively charged membrane of Gram-negative bacteria, disrupt the membrane and allow binding with other anionic targets, thus killing the bacteria in the process (Hancock and Scott, 2000). Found mainly in the mucous layer covering and protecting epithelial tissues, these peptides are produced by epithelial cells, neutrophils and intestinal Paneth cells (Dempsey *et al.*, 2003; Hancock and Scott, 2000).

Other than killing the pathogens directly through phagocytosis, phagocytes also induce the next phase of innate immunity by causing inflammation. Inflammation recruits other immune cells to the site of infection and is dependent on the cytokines and chemokines produced by phagocytes (Dempsey *et al.*, 2003; Takeuchi and Akira, 2010). Cytokines such as TNF-α, interleukin (IL)-1β and IL-6 and chemokines such as CCL2 and CXCL8 are produced during the process. The production of cytokines and chemokines are dependent on the recognition of pathogen-associated molecular patterns (PAMPs) – conserved and repetitive surface structures on pathogens – by germline-encoded pattern recognition receptors (PRRs) (Dempsey *et al.*, 2003; Takeuchi and Akira, 2010).

There are 4 classes of PRR families identified and they include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). They can be further classified into transmembrane (TLRs and CLRs) and cytosolic (RLRs and NLRs) PRRs (Takeuchi and Akira, 2010). Phagocytic receptors such as scavenger receptors, mannose receptor and Dectin-1 are also PRRs. Engagement of PRRs triggers various intracellular signaling cascades which

eventually leads to transcriptional and translational expression of inflammatory cytokines and chemokines, helping in the clearance of infections (Takeuchi and Akira, 2010).

1.1.3 Complement system

The complement system plays an important role in the humoral arm of innate immunity by helping to defend against bacterial infections, linking the innate and adaptive immune systems and clearing of immune complexes and products of inflammation (Walport, 2001). First discovered in 1896, the complement system is a heat-labile component of the serum and complements the antibacterial properties of heat-stable antibodies. To date, more than 30 complement proteins are found which consist of both plasma and membrane associated proteins (Walport, 2001). These proteins formed a series of proteolytic reactions, amplifications and complex formations to mediate their functions.

There are three different pathways for the activation of the complement system and they are namely the classical pathway, the lectin pathway and the alternative pathway (Figure 1.1) (Dunkelberger and Song, 2010; Walport, 2001). The three pathways differ from one another at the initiation step in which they recognize different targets for activation (Dunkelberger and Song, 2010; Walport, 2001). For the classical pathway, it is antibody-dependent and is activated upon the binding of C1q to immune complexes (Duncan and Winter, 1988). The lectin pathway is initiated through the binding of mannose-binding lectin (MBL) on specified carbohydrate patterns on microbial pathogens (Fujita *et al.*, 2004). Lastly, the alternative pathway is constantly activated but at low levels. Full activation of the alternative pathway is prevented by surface-bound and soluble regulators on host cells. However, this inhibition is absent on foreign cells which allows their removal from the body (Ricklin *et al.*, 2010; Thurman and Holers, 2006).

Despite the differences between the three pathways, all of them converge in the formation of the C3 convertase (C4bC2a for classical and lectin pathways and C3bBb for alternative pathway) (Dunkelberger and Song, 2010; Walport, 2001). This allows the clearance of the pathogens via different means such as phagocytosis of the opsonized pathogens by phagocytes, production of anaphylatoxins for the recruitment of inflammatory cells and direct lysis of the pathogens through the formation of the membrane attack complex (MAC) (Dunkelberger and Song, 2010; Walport, 2001).

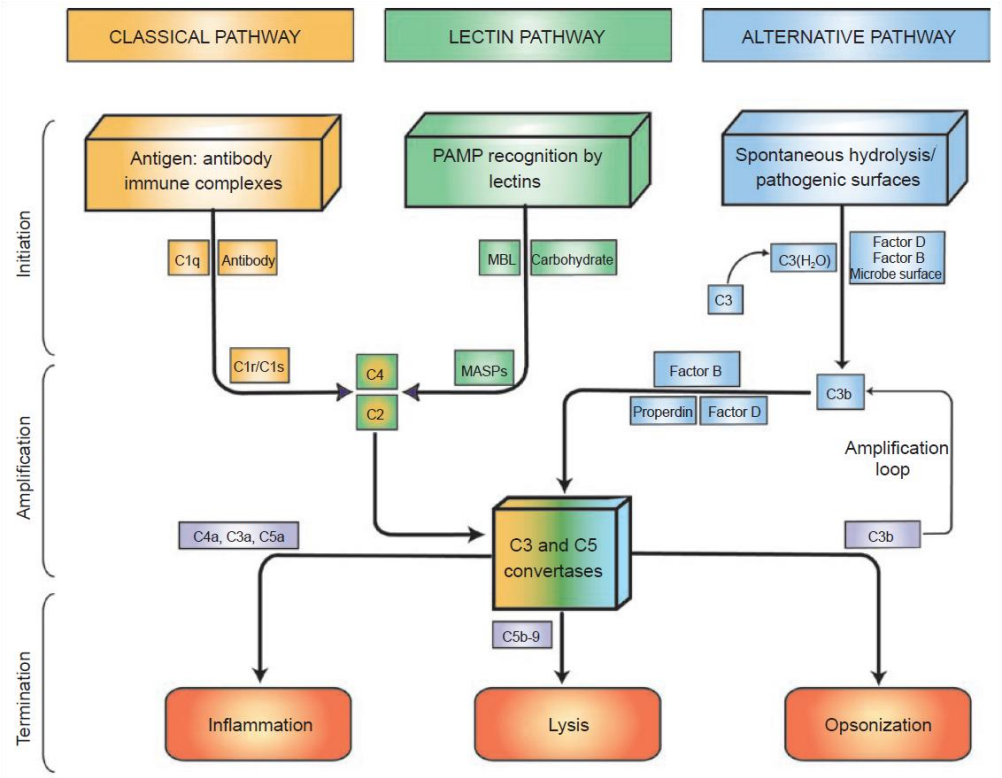


Figure 1.1. The complement pathways. The complement system can be activated via three different pathways namely classical, lectin and alternative pathways. Each pathway differs from each other mainly at the initiation stage in which different ligands are required for activation. In the classical pathway, the recognition of clustered Fc regions of antibodies (IgG or IgM) by C1q activates the pathway. For the lectin pathway, mannose-binding lectin (MBL) binds to specific carbohydrate structures on microbes and activates the pathway. The alternative pathway consists of low level constitutive complement activation which is regulated by host cells. In the absence of such regulators on pathogens, the pathway is fully activated and caused the removal of the pathogens. All three pathways converged in the formation of the C3 convertase C4bC2a for classical and lectin pathways and C3bBb for alternative pathway. This leads to different modes of action such as inflammation, opsonization and lysis which aid in the removal of invading threat. The figure is adapted from Dunkelberger and Song, 2010.

1.1.4 Classical complement pathway

The classical pathway is the first complement pathway to be described and plays an important role in antimicrobial defense (Walport, 2001). It can bind to pathogens directly or indirectly after recognition by antibodies. In addition, the classical pathway is known to be involved in immune tolerance (Lu *et al.*, 2008) and rejection in xenograft transplantation (Sacks and Zhou, 2012).

The pathway is initiated by C1, a 790 kD pentameric complex. C1 complex ($C1qC1r_2C1s_2$) consists of a recognition subunit C1q and a catalytic subunit calcium-dependent tetramer $C1r_2C1s_2$ in a ratio of 1:2:2 (Arlaud *et al.*, 2002). The C1r and C1s molecules exist as proenzymes which are single-chained serine proteases and they exist as two-chain proteases after activation (Arlaud *et al.*, 2002).

Upon binding to the fragment crystallizable (Fc) regions of the antibody-antigen complex by C1q, a conformation change is induced in the C1complex and this results in the autoactivation of C1r. Subsequently, the active C1r cleaves and activates C1s. Activated C1s then cleaves C4, forming C4a and C4b. C4b binds convalently to the bacteria surface and recruits C2 which is cleaved by C1s. This produces C2a and C2b. C2a is a serine protease and together with C4b, they form the C3 convertase (C4bC2a). The C3 convertase cleaves C3 into C3a and C3b and the resulting C3b binds to C4b in the C3 convertase, forming the C5 convertase (C4bC2aC3b). C3b also acts as the acceptor site for C5 in C5 convertase which cleaves C5 into C5a and C5b. This initiates the formation of the MAC with C5b binding to C6 and the C5bC6 complex will then recruit C7. The binding of C7 to C5bC6 complex induces a conformation change in the molecule, exposing the hydrophobic region and allow the insertion of the complex (C5bC6C7) into the membrane of the bacteria. C8 is subsequently recruited and similar to C7, the association of C8 to C5bC6C7 complex exposes its hydrophobic site and causes the insertion of C8 into the membrane. The resulting complex (C5bC6C7C8) then recruits and induces the polymerization of 10 - 16 C9 molecules into the

membrane of the bacteria, forming the terminal MAC. This results in pore formation on the bacteria causing the eventual lysis of the bacteria.

During the process of complement activation, other molecules formed as a result of proteolytic cleavages are also involved in the host defense against the pathogens (Walport, 2001). For example, C3a, C4a and C5a are anaphylatoxins which are involved in the chemotaxis and activation of leukocytes to the affected sites (Walport, 2001; Zhou, 2012). The initiation and regulation of inflammation by anaphylatoxins assist in the elimination of the infection (Zhou, 2012). Furthermore, opsonins such as C3b and its proteolytic fragments and C4b are deposited on the surfaces of the pathogens. This enhances the removal of the pathogens by phagocytes through phagocytosis (Underhill and Ozinsky, 2002).

1.2 C1q

1.2.1 Structure of C1q

C1q is a 460kD macromolecule made up of 18 polypeptides consisting of 6 A, 6 B and 6 C chains (Figure 1.2) (Lu *et al.*, 2008). Each polypeptide consists of a collagenous N-terminal and a globular C-terminal (Kishore and Reid, 1999). The A and B chains dimerize with the help of a disulphide bond at the N-terminal ends to form a heterodimer (Reid and Porter, 1976; Yonemasu and Stroud, 1972). Similarly, 2 C chains form a homodimer through the formation of a disulphide bond at the N-termini (Reid and Porter, 1976; Yonemasu and Stroud, 1972). The A-B heterodimer and a single C chain form a triple helix at the collagen-like regions and this brings 2 such structures (ABC heterotrimer) together through the C-C homodimer (Lu *et al.*, 2008; Reid and Porter, 1976). Further interactions at the collagenous N-termini allow three ABC-CBA structures to form the "stalk" of the functional C1q molecule (Lu *et al.*, 2008; Reid and Porter, 1976). Due to disruptions in the Gly-Xaa-Yaa motifs of the collagenous domains, the "stalk" diverges into 6 "arms" of heterotrimeric ABC structures followed by their globular heads (Reid and Porter, 1976). For the globular heads, they exist as compact, almost spherical heterotrimeric assemblies which are held together by non-polar interactions (Gaboriaud *et al.*, 2003). This explains the ability of C1q to recognize a wide range of ligands (Gaboriaud *et al.*, 2003; Kojouharova *et al.*, 2003). Under the electron microscope, the functional C1q molecule is viewed as a "bundle-of-tulips" (Knobel *et al.*, 1975).

In human, the C1q genes are found highly clustered within a genomic region of approximately 25 kb in chromosome 1 in the order of C1qA-C1qC-C1qB (Sellar *et al.*, 1991). The clustered genes allowed the coordinated expressions of these genes under basal and induced conditions (Chen *et al.*, 2011). This accounts for the 1:1:1 ratio for the three C1q subunits in the assembly of the functional C1q molecule.

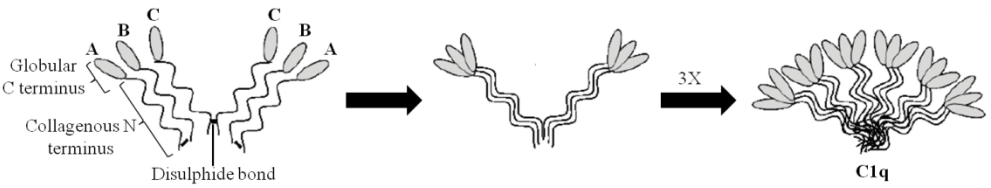


Figure 1.2. Formation of functional C1q molecule. Each chain consists of a collagenous N terminus and globular C terminus. Disulphide bonds are formed between the A and B chains and 2 C chains giving rise to A-B and C-C hetero- and homodimers respectively. Further interactions at the collagenous and globular domains bring the A, B and C chains together, forming a 6-chain structure. More interactions at the collagenous N termini allows three such structures to come together to form the functional C1q molecule. The figure is adapted from Lu *et al.*, 2008.

1.2.2 Role of C1q in complement activation

As the recognition subunit in the classical pathway, C1q binds to the Cγ2 and Cμ3 domains of the fragment crytallizable (Fc) regions of immunoglobulin (Ig) G or IgM bounded on immune complexes respectively (Cooper, 1985; Kishore and Reid, 1999). This is achieved through its heterotrimeric globular heads with all three chains involved though the globular region of B chain may have a more central role in C1q-IgG interaction (Kojouharova *et al.*, 2004). C1q binds aggregated IgG approximately 10,000-fold more efficiently than soluble monomeric IgG accounting for the activation of C1 complex on immune complexes. Among the IgG subclasses, C1q has the strongest interaction with IgG3 followed by IgG1 and IgG2 whereas IgG4 shows minimal binding to C1q (Cooper, 1985; Kishore and Reid, 1999).

Other than activating the classical pathway through antibody-dependent mechanism, C1q is able to bind to a wide range of other ligands to activate the complement system (Table 1) (Cooper, 1985). These include Gram-positive and Gram-negative bacteria, viruses, cellular and subcellular structures from damaged cells, apoptotic cells, proteins, carbohydrates, lipids and polyanions. There are no common structures in these diverse ligands and some of them can activate the complement system more effectively than Igs. C1q binds these

ligands mainly through its globular heads though the collagen tails are involved occasionally (Cooper, 1985; Kishore and Reid, 1999).

Table 1.1. Activators of the classical complement pathway. The table is adapted from Cooper, 1985

Immune activators
• Antigen–antibody complexes containing IgM or IgG
Non-immune activators
• Various bacterial strains (*Escherichia coli, Salmonella and Klebsiella*)
• Mycoplasma (*Mycoplasma pneumoniae*)
• Various viruses (Sindbis, Newcastle disease and Epstein–Barr virus) and retroviruses [human immunodeficiency virus (HIV)]
• Parasites (*Schistosoma mansoni* and *Trypanosoma brucei*)
• Cellular and subcellular membranes, and apoptotic cells
• Proteins [C-reactive protein (CRP), long pentraxin PTX3, myelin, β-amyloid peptide, serum amyloid P component and prion protein)
• Oligosaccharides and polysaccharides
• Lipids (lipid A, cardiolipin and enzymatically modified form of low-density lipoprotein)
• Polyanions (heparin and DNA)

1.2.3 Clearance of apoptotic cells by C1q

C1q is involved in the clearance of apoptotic cells through direct opsonization or indirect opsonization via complement activation (Lu *et al.*, 2008; Nayak *et al.*, 2010). This is mainly due to the ability of C1q to bind to diverse ligands leading to various effector mechanisms in removing the apoptotic cells.

1.2.3.1 Direct opsonization of apoptotic cells

During apoptosis, cells will undergo multiple changes which include blebbing and exposure of intracellular molecules on their surfaces (Elmore, 2007). C1q binds to apoptotic blebs (Korb and Ahearn, 1997) and this is mediated through the interaction between its globular heads and surface-

exposed DNA and phosphatidylserine (PS) on apoptotic blebs (Paidassi *et al.*, 2008a; Paidassi *et al.*, 2008b). C1q then binds to calreticulin/CD91complex on the cell surfaces of phagocytes through its collagenous tails to calreticulin (Ogden *et al.*, 2001). The apoptotic cells are eventually removed by phagocytes via CD91-mediated macropinocytosis (Ogden *et al.*, 2001).

1.2.3.2 Opsonization of apoptotic cells through complement activation

C1q can also enhance apoptotic cell clearance by complement activation. One of the mechanisms is mediated through the binding of C1q to polyclonal IgM on apoptotic cells. This results in C3 deposition on apoptotic cells which enhances clearance by phagocytes (Ogden *et al.*, 2005; Zwart *et al.*, 2004). However, IgM binds only to late but not early apoptotic cells (Zwart *et al.*, 2004).

Many molecules exposed on apoptotic cells can bind to C1q directly or indirectly, activate the complement system and results in deposition of opsonins on apoptotic cells. On apoptotic cells, C1q binds directly to DNA (Jiang *et al.*, 1992) and PS (Mevorach *et al.*, 1998) whereas it binds indirectly to phosphorylcholine and chromatin through CRP and serum amyloid protein (SAP) (Lu *et al.*, 2008). Complement activation by C1q is likely to be more effective in removing apoptotic cells as a single molecule can lead to deposition of multiple C3 molecules provided C3 activation does not lead to excessive inflammation and tissue damage (Lu *et al.*, 2008).

1.2.4 Immune modulation of cells associated with C1q

C1q can regulate the functions and activities of various immune cells such as macrophages, DCs and lymphocytes (B and T cells) (Lu *et al.*, 2008; Nayak *et al.*, 2010). This leads to an effective immune system due to the coordination of these immune cells.

1.2.4.1 Regulation of macrophages

Macrophages are scavengers in our body and they interact with both foreign and host cells. Besides phagocytic activities, macrophages are able to secrete inflammatory mediators to aid in the immune response. C1q is able to interact with soluble and cellular molecules of both foreign and host cells to affect the activities of macrophages (Lu *et al.*, 2008).

Foreign cells such as bacteria are targets for the immune system. C1q is shown to bind to and directly opsonizes *Listeria monocytogenes* for enhanced phagocytosis by macrophages (Alvarez-Dominguez *et al.*, 1993). This process also results in increased cytotoxicity to the bacteria due to upregulation of IFNγ-induced superoxide and nitric oxide production by macrophages (Alvarez-Dominguez *et al.*, 2000). In additional, C1q can induce complement activation on *Streptococcus pneumonia* in which direct binding of C1q to the bacteria is not required (Kang *et al.*, 2006). Both C1q and *S. pneumonia* bind to lectin receptor SIGN-R1 and the binding of C1q to SIGN-R1 causes direct complement activation, leading to C3 deposition on *S. pneumonia* (Kang *et al.*, 2006).

C1q can also affect macrophages directly and help in the removal of pathogens. Soluble C1q, produced endogenously and increased by Lipid A (Jiang *et al.*, 1996a), is shown to upregulate the expression of tumor necrosis factor (TNF)-α receptor in macrophages (Jiang *et al.*, 1996b). This enhances autocrine signaling by TNF-α, resulting in increased nitric oxide production though nitric oxide synthase and causes more cytotoxicity to the bacteria (Jiang *et al.*, 1996a; Jiang *et al.*, 1996b). Furthermore, soluble C1q induces TNF-α and C3 production in macrophages which helps in enhanced phagocytosis (Bajtay *et al.*, 2000). On the other hand, immobilized C1q has been shown to engage CD93 on macrophages directly and augment Fc receptor- and CR1-dependent phagocytosis in macrophages (Bobak *et al.*, 1988; Bobak *et al.*, 1987; Nepomuceno *et al.*, 1997).

As mentioned earlier in 1.2.3, clearance of apoptotic cells can be enhanced by C1q through direct and indirect opsonization (Lu *et al.*, 2008; Nayak *et al.*, 2010). This allows efficient phagocytosis by macrophages and maintains self tolerance.

1.2.4.2 Regulation of DCs

DCs are sentinels of the immune system and they are involved in detecting pathogens and subsequent activation of adaptive immunity. C1q is able to modulate their functions through apoptotic cells or during their differentiation from monocytes.

C1q-mediated opsonization is required for the uptake of apoptotic cells by DCs although questions remain if C1q alone can mediate apoptotic cell uptake (Baruah *et al.*, 2006; Fraser *et al.*, 2009; Nauta *et al.*, 2004). Furthermore, C1q-opsonized apoptotic cells modulate cytokine production by DCs (Baruah *et al.*, 2006; Fraser *et al.*, 2009; Nauta *et al.*, 2004). A study showed increased IL-12p70 production by DCs when stimulated with both LPS and C1q-opsonized apoptotic cells (Baruah *et al.*, 2006). However, two other studies showed more similar results. The first study indicated increased TNF-α, IL-6 and IL-10 but unchanged IL-12p70 levels in the presence of C1q-opsonized apoptotic cells (Nauta *et al.*, 2004). Another study showed LPS and C1q-opsonized late apoptotic cells induced TNF-α, MCP-1 and IL-10 but inhibit MIP-1α production (Fraser *et al.*, 2009).

Besides affecting DCs through C1q-opsonized apoptotic cells, C1q can regulate the differentiation of DC from monocytes (Castellano *et al.*, 2007; Csomor *et al.*, 2007; Fraser *et al.*, 2009; Teh *et al.*, 2011). DCs differentiated in the presence of soluble C1q gave rise to immature cells with high phagocytic capacity and low surface expression of CD80, CD83 and CD86 (Castellano *et al.*, 2007). No difference in phagocytic capacity was observed between these DCs and normal DCs. When exposed to LPS, there was a significant inhibition in the production of TNF-α, IL-6 and IL-10 accompanied with limited

upregulation of CD80, CD83 and CD86. These DCs also had impaired ability in stimulating allogeneic T cells and IFNγ production by these T cells (Castellano *et al.*, 2007).

For DCs differentiated on immobilized C1q, there are conflicting results from different studies (Csomor *et al.*, 2007; Fraser *et al.*, 2009; Teh *et al.*, 2011). One study showed immobilized C1q induced maturation of DCs as indicated by the upregulation of co-stimulatory molecules CD83, CD86, CCR7 and MHC II (Csomor *et al.*, 2007). Furthermore, these DCs produced more IL-10, IL-12 and TNF-α and stimulated both proliferation and IFNγ production of allogeneic T cell proliferation (Csomor *et al.*, 2007). However, another recent study shows contrasting results for DCs differentiated on immobilized C1q (Teh *et al.*, 2011). These DCs, both immature and mature, had similar expression of cell surface immune molecules like MHC class I, MHC class II, CD40, CD80, CD83, CD86 and CCR7 as normal DCs though they phagocytosed more apoptotic cells than normal DCs. When stimulated, they showed increased IL-10 level but reduced IL-12 and IL-23 levels. There was reduced induction of Th1 and Th17 T cells when incubated with allogeneic T cells and a concomitant decrease in the production of IFNγ and IL-17 by these T cells (Teh *et al.*, 2011). Another study also showed similar results when DCs differentiated on immobilized C1q produced less cytokines such as IL-1β, TNF-α, MCP-1, IL-6 and IL-10 as compared to control DCs (Fraser *et al.*, 2009).

1.2.4.3 Regulation of B and T cells

B and T cells are important regulators of adaptive immunity. Both cell types express C1q receptors and C1q has been shown to regulate the functions of both lymphocytes (Nayak *et al.*, 2010).

Aggregated C1q enhanced Ig production in B cells through its collagen tail (Daha *et al.*, 1990). The increased Ig production by C1q was also validated in another study using activated B cells (Young *et al.*, 1991). Using C1q

knockout mice, the absence of C1q decreased negative selection of autoreactive conventional B cell and increased positive selection of B1b B cells and IgM autoantibodies by intracellular antigens exposed during apoptosis, leading to reduction in B cells tolerance (Ferry *et al.*, 2007).

C1q was shown to inhibit proliferation and activation of T cells (Chen *et al.*, 1994). In C1q knockout mice, IFNγ production was reduced in antigen specific T cells (Cutler *et al.*, 1998). This led to abnormal humoral response and production of IgG2a and IgG3was impaired (Cutler *et al.*, 1998). However, in humans, immobilized C1q did not affect IFNγ level but it inhibited IL-4 production and enhanced IL-10 production in T cells (Lu *et al.*, 2007).

1.2.4.3 Regulation of other immune cells

C1q has also been shown to modulate the activities of other immune cells. It can activate microglial cells and attenuate their proliferation (Farber *et al.*, 2009). Furthermore, C1q enhanced the uptake of apoptotic neurons and its blebs by microglial cells and suppressed the subsequent production of proinflammatory cytokines such IL-1α, IL-1β, IL-6 and TNF-α by LPS stimulation (Fraser *et al.*, 2010).

For platelets, C1q alone can cause their aggregation and activation (Peerschke *et al.*, 1993). It also augments platelet aggregation and activation in the presence of immune complexes during tissue damage and inflammation (Peerschke and Ghebrehiwet, 1997). C1q is shown to induce superoxide production in neutrophils through its collagen tail (Tenner and Cooper, 1982). A study indicates that C1q is required for IL-6 production by mast cells when stimulated with immune complexes (Edelson *et al.*, 2006). Furthermore, the proliferation of mast cells was also inhibited by C1q due to decreased DNA synthesis (Ghebrehiwet *et al.*, 1995).

1.2.5 Additional functions mediated by C1q

C1q also regulates various processes such as adhesion, chemotaxis, pregnancy and cancer formation (Lu *et al.*, 2008; Nayak *et al.*, 2010). Many cell types are influenced by C1q and these cells mainly have C1q receptors.

The interaction of the C1q collagen tail and its receptor enhanced fibroblast adhesion (Bordin *et al.*, 1990). C1q also induces adhesion and spreading of human dermal microvascular endothelial cells (HDMVEC) which required the cooperation of C1q receptors and β_1 integrins (Peerschke *et al.*, 1996). Immune complexes containing C1q enhanced endothelial cells adhesiveness to leukocytes by increasing endothelial expression of adhesion molecules such as E-selectin, intercellular cell adhesion molecule (ICAM)-1 and vascular intercellular adhesion molecule 1 (VCAM)-1 (Lozada *et al.*, 1995). In addition, C1q has been shown to regulate adhesion of monocytes (Ma *et al.*, 2012) and platelets (Peerschke and Ghebrehiwet, 1997; Peerschke *et al.*, 1993).

C1q is able to regulate chemotaxis of various cell types which includes DCs (Vegh *et al.*, 2006), eosinophils (Kuna *et al.*, 1996), fibroblasts (Oiki and Okada, 1988), mast cells (Ghebrehiwet *et al.*, 1995) and neutrophils (Leigh *et al.*, 1998). The process is highly dependent on C1q receptors present on these cells (Ghebrehiwet *et al.*, 1995; Kuna *et al.*, 1996; Leigh *et al.*, 1998; Oiki and Okada, 1988; Vegh *et al.*, 2006). It is also shown that C1q was involved in chemokinesis of mast cells (Ghebrehiwet *et al.*, 1995).

During pregnancy, the presence of C1q on the surface of decidual endothelial cells (DECs) acts as an intercellular molecular bridge between DECs and endovascular trophoblasts (Bulla *et al.*, 2008). C1q, together with gC1qR and β_1 integrins, helps in the adhesion and migration of trophoblasts, allowing trophoblasts to invade the deciduas during placentation (Agostinis *et al.*, 2010). C1q knockout mice exhibited defective invasion by trophoblasts and resulted in restricted embryonic growth (Agostinis *et al.*, 2010).

C1q can modulate the proliferation and cell death of cancer cells. In addition of inhibiting the proliferation of mast cells (Ghebrehiwet *et al.*, 1995), microglial cells (Farber *et al.*, 2009) and T cells (Chen *et al.*, 1994), C1q has an anti-proliferative effect on several malignant cell lines (Ghebrehiwet *et al.*, 1990). C1q activates tumor suppressor WOX1 and destabilizes cell adhesion in prostate cancer cells, leading to apoptosis of the cells (Hong *et al.*, 2009). The same study also indicated C1q induced similar WOX1-mediated apoptosis in breast cancer cells and neuroblastoma cells (Hong *et al.*, 2009).

1.2.6 Diseases associated with C1q

Given that C1q plays multiple roles in many different functions, immune or non-immune, and affects diverse cell types, it is not surprising that C1q is associated with a wide array of diseases. These diseases include autoimmune, cardiovascular, neurological and infectious diseases in which C1q plays a direct or indirect role in their manifestations.

1.2.6.1 SLE

SLE is a systemic autoimmune disease which affects multiple organs. It has diverse clinical symptoms and these include rash, arthritis, anaemia, thrombocytopenia, serositis, nephritis, seizure and psychosis (Rahman and Isenberg, 2008). Most of the patients are predominantly females but it is not known if it is due to female hormones having a critical role in causing the disease, the protective effects of male hormones or the effects of genes on the X chromosome (Rahman and Isenberg, 2008).

In contrast to the confusion over the predominant nature of SLE in females, great strides have been made in the understanding of the disease. SLE is characterized by the production of anti-nuclear autoantibodies, formation and deposition of immune complexes and extensive tissue damage (Lewis and Botto, 2006; Rahman and Isenberg, 2008). Many studies involving different

populations have also shown the polygenic nature of SLE (Deng and Tsao, 2010). Out of these genes, hereditary deficiencies in early complement proteins of the classical pathway have been shown to be a strong risk factor for SLE (Lewis and Botto, 2006; Truedsson *et al.*, 2007).

Patients with hereditary complement deficiencies tend to have an early onset of SLE and have more severe disease presentations (Pickering *et al.*, 2000). Furthermore, the female to male predominance in these patients is lost as compared to the majority of SLE patients (Pickering *et al.*, 2000). A hierarchal association is observed that the position of the early complement factors in the classical pathway affects the susceptibility factor for SLE in the absence of such factors (Lewis and Botto, 2006). C1q has the strongest association with SLE in which C1q deficiency has a 93% risk of developing SLE. This is followed by a risk factor of 75% and 10% for C4 and C2 deficiency respectively (Lewis and Botto, 2006).

As C1q is required for the clearance of apoptotic cells (Korb and Ahearn, 1997; Ogden *et al.*, 2001; Quartier *et al.*, 2005), the absence of C1q caused the accumulation of apoptotic cells and exposure of autoantigens (Casciola-Rosen *et al.*, 1994). This eventually leads to the formation of autoantibodies observed in SLE patients (Rahman and Isenberg, 2008). Furthermore, C1q deficiency resulted in impaired clearance of immune complexes (Schifferli *et al.*, 1986) which explained the presence of such complexes in different tissues. Although C1q deficient individuals are rare, many SLE patients displayed low levels of C1q during renal flares (Sinico *et al.*, 2009; Tsirogianni *et al.*, 2009) and this further strengthens the role of C1q in SLE.

Despite its seemingly important role in the prevention of SLE, C1q has also been implicated in the inflammatory stage of the disease (Cook and Botto, 2006; Lewis and Botto, 2006; Rahman and Isenberg, 2008; Truedsson *et al.*, 2007). Immune complexes, deposited or formed via the binding of autoantibodies to autoantigens, found in SLE patients are able to activate the classical complement pathway via C1q. This results in the generation of

anaphylatoxins (C3a and C5a), recruitment of inflammatory cells and release of inflammatory mediators, causing tissue injury and enhancing the inflammation process (Cook and Botto, 2006; Rahman and Isenberg, 2008). Thus, C1q acts as a double edged sword in SLE.

To establish the association between C1q deficiency and SLE, Botto and colleagues (1998) first generated C1q-deficient mice in which the *C1qa* gene was disrupted by gene targeting. These $C1qa^{-/-}$ mice did not have detectable levels of circulating C1q protein and haemolytic activity was also absent thus assimilating C1q-deficient patients (Botto *et al.*, 1998). These mice displayed highly similar phenotypes as C1q-deficient patients and the phenotypes include anti-nuclear autoantibodies, glomerulonephritis with immune complex deposits and presence of apoptotic bodies in glomeruli (Botto *et al.*, 1998). Thus, this supports the hypothesis that C1q deficiency leads to SLE.

However, the development of the lupus-like phenotypes was dependent on the mice strain used as these phenotypes were found mainly in 129/Ola x C57BL/6 hybrid strains but not but not pure 129/Ola inbred mice (Botto *et al.*, 1998). Another study has also validated that background genes played a significant role in the development of the autoimmune disease in the absence of C1q (Mitchell *et al.*, 2002). This is hardly surprising given the polygenic nature of lupus in both human (Deng and Tsao, 2010) and mice (Morel *et al.*, 1994; Vyse and Kotzin, 1998).

1.2.6.2 Cardiovascular diseases

C1q and its associated complement activation are involved in cardiovascular diseases. Complement proteins including C1q were found upregulated at mRNA and protein levels in human heart at areas of old and recent myocardial infarction (Yasojima *et al.*, 1998). Similarly, C1q accumulation was observed in canine model of myocardial infarction with the recruitment of neutrophils in C1q-rich ischemic regions (Rossen *et al.*, 1985).

Furthermore, C1q can bind to CRP, which was found in infarcted heart tissues of patients who died of acute myocardial infarction (Lagrand *et al.*, 1997), leading to complement activation (McGrath *et al.*, 2006). These studies suggest that complement activation aggravates the damage during myocardial infarction.

Beside the involvement of C1q in myocardial infarction, it is also linked to atherosclerosis. C1q can bind modified low density lipoproteins and activate the complement system (Biro *et al.*, 2007). In addition, C1q can associate with endothelial cells, trigger complement activation and worsen inflammation of the endothelium (Yin *et al.*, 2007). Further complement activation can occur through vascular injury during atherosclerosis which is due to platelet activation after binding to C1q (Peerschke *et al.*, 2009). These factors contribute to the pathogenesis of atherosclerosis by complement activation.

1.2.6.3 Neurological diseases

C1q plays contrasting roles in the central nervous system (CNS). Firstly, it is involved in synapse pruning during brain development (Stevens *et al.*, 2007). C1q knockout mice exhibited increased epilepsy due to the failure to prune excessive synapses during development (Chu *et al.*, 2010). Microglial cells are phagocytes of the CNS and help in removing debris during development and injury. C1q has a protective role in which it enhances the removal of apoptotic cells by microglial cells and suppresses proinflammatory cytokines production during early stages of cell death (Fraser *et al.*, 2010). A study showed the involvement of C1q in clearing extracellular neuromelanin and degenerated neurons from the substantia nigra in Parkinson disease, preventing inflammation (Depboylu *et al.*, 2011).

On the other hand, C1q and complement activation are involved in many neurological diseases contributing to the pathology of the diseases by causing inflammation and tissue injury. C1q can activate microglial cells and

induce the production of proinflammatory cytokines (Farber *et al.*, 2009). In the rat model of global ischemia, biosynthesis of C1q by microglial cells were upregulated and C1q was also found in the cerebrospinal fluid (Schafer *et al.*, 2000). In humans, C1q, C3c and C4d depositions were detected in ischemic lesions (Pedersen *et al.*, 2009). This implies that proinflammatory activities of C1q may contribute to the pathology of cerebral ischemia (Pedersen *et al.*, 2009; Schafer *et al.*, 2000). Alzheimer's disease is a neurodegenerative disease characterized by deposition of β-amyloid plaques and neurofibrillary tangles. In the brains of patients with Alzheimer's disease, expression of C1q and other complement proteins were upregulated as compared to controls (Yasojima *et al.*, 1999). C1q has been shown to bind to the primary constituents of the plaques and tangles, β-amyloid (Rogers *et al.*, 1992; Tacnet-Delorme *et al.*, 2001) and tau (Shen *et al.*, 2001) respectively, and activate the complement system. Furthermore, C1q is involved in mediating synapse loss in glaucoma and possibly other CNS neurodegenerative diseases (Stevens *et al.*, 2007).

C1q is also involved in the pathogenesis of scrapie diseases (Klein *et al.*, 2001; Mabbott *et al.*, 2001). C1q knockout mice showed protection against transmissible spongiform encephalopathy after exposure to scrapie (Klein *et al.*, 2001; Mabbott *et al.*, 2001). The prion protein can bind to C1q and activate the complement system (Mitchell *et al.*, 2007). This may help in the transmission of the protein to lymphoid tissues by attaching to C3 fragments and cause the eventual attachment to follicular DCs (Mitchell *et al.*, 2007). In addition, the absence of C1q prevents the uptake of prion protein by conventional DCs and the subsequent accumulation on follicular DCs, delaying the development of the disease (Flores-Langarica *et al.*, 2009).

1.2.6.4 Infectious diseases

As part of the first line of defense against pathogens, C1q helps in removing pathogens through various means such as complement activation and opsonization for the uptake by phagocytes (Lu *et al.*, 2008). Thus, the absence of C1q would lead to increased host susceptibility to infections. C1q knockout

mice showed increased susceptibility to *Plasmodium* (Taylor *et al.*, 2001), polymicrobial peritonitis (Celik *et al.*, 2001) and *Salmonella* (Warren *et al.*, 2002) infections.

C1q can control infections through additional means other than the complement system. An example is that C1q enhanced the potency of antibodies against West Nile virus by modulating the stoichiometric requirements for neutralization (Mehlhop *et al.*, 2009). This helps to prevent antibody-dependent enhancement (ADE) infection of the cells as C1q reduced the number of antibodies needed for neutralization below that of the threshold required for ADE, preventing exacerbation of the disease (Mehlhop *et al.*, 2009).

1.2.6.5 Other diseases

Complement activation by C1q is also proposed to be involved in many inflammatory diseases. Small leucine-rich repeat proteins were shown to bind C1q and activate the complement (Sjoberg *et al.*, 2005; Sjoberg *et al.*, 2009). Thus, it is suggested that these proteins which are found in the extracellular matrix may mediate chronic inflammation in diseases such as rheumatoid arthritis, osteoarthritis and chronic obstructive lung disease (Sjoberg *et al.*, 2009).

During aging, serum C1q concentration and expression of C1q in various tissues were increased (Naito *et al.*, 2012). This is also accompanied with increased Wnt signaling which is activated by C1q. Thus, this leads to impaired muscle regeneration in aged mice and could be possibly involved in other aging-related phenotypes.

1.3 C1q receptors

To mediate the various functions of C1q, it is difficult to imagine that only one receptor is involved. There are many proteins, both cell surface and intracellular, that help in triggering or enhancing different cellular functions by binding to C1q and act as C1q receptors. gC1qR and cC1qR are two well known receptors that are ubiquitously expressed in cells and bind to the globular head and collagen tail of C1q respectively (Lu *et al.*, 2008).

1.3.1 g1qR/p33/C1qBP

gC1qR, also known as p33 or C1qBP, is a 33 kD acidic protein and has a pI of 4.74 (Lu *et al.*, 2008). The presence of a high affinity receptor recognizing the globular heads of C1q was first reported in fibroblasts (Bordin and Page, 1989) and was subsequently characterized in Raji cells (Ghebrehiwet *et al.*, 1994). C1q binds to residues 74 - 95 of gC1qR and the interactions between C1q and globular heads of C1q are thought to be primarily ionic interactions though other interactions may be possible (Ghebrehiwet *et al.*, 1994). To date, gC1qR is found on the surfaces of B cells (Ghebrehiwet *et al.*, 1994), DCs (Vegh *et al.*, 2006), eosinophils (Kuna *et al.*, 1996), fibroblasts (Bordin and Page, 1989), mast cells (Ghebrehiwet *et al.*, 1995), neutrophils (Leigh *et al.*, 1998) and platelets (Peerschke *et al.*, 1994).

However, gC1qR lacks a transmembrane domain and is primarily found in the mitochondria (Dedio *et al.*, 1998) with some nucleus localization detected (Matthews and Russell, 1998). However, several mechanisms may explain the presence of gC1qR on cell surfaces. gC1qR may associate with other transmembrane proteins, leading to surface expression and signaling via these transmembrane proteins (Ghebrehiwet *et al.*, 2001). A study also showed that mitochondria targeting of gC1qR can be affected by adding a small tag to the N-terminus of gC1qR, resulting in localization on cell surface and in endoplasmic reticulum(van Leeuwen and O'Hare, 2001). This implies that the

association of gC1qR with other proteins might interfere with it mitochondria localization.

1.3.2 cC1qR/Calreticulin

cC1qR, also known as calreticulin, is a 60 kD acidic protein and has a pI of 4.29 (Lu *et al.*, 2008). It is the first receptor suggested for C1q(Dickler and Kunkel, 1972) and binds to the collagen tail of C1q (Arvieux *et al.*, 1984). Subsequently, partial sequence of the receptor was determined (Malhotra *et al.*, 1993) and combined with other studies (Eggleton *et al.*, 1994), calreticulin is identified as cC1qR. Calreticulin has three domains namely N-domain, P-domain and C-domain (Michalak *et al.*, 1999) and the collagen tail of C1q was determined to bind at the intersection between the N- and P- domains (Stuart *et al.*, 1997). Furthermore, calreticulin is also known as the collectin receptor as it binds MBL, surfactant protein (SP)-A and SP-D (Malhotra *et al.*, 1990). It is ubiquitously expressed and can be found in cells such as eosinophils (Kuna *et al.*, 1996), mast cells (Ghebrehiwet *et al.*, 1995), neutrophils (Eggleton *et al.*, 1994) and platelets (Peerschke and Ghebrehiwet, 1997; Peerschke *et al.*, 1993) to mediate the effects of C1q.

Similar to gC1qR, calreticulin is an intracellular protein and localizes to the lumen of endoplasmic reticulum (ER) due to C-terminal KDEL ER retrieval signal. The main functions of calreticulin in the endoplasmic reticulum are regulating calcium homeostasis and ensuring correct protein folding by acting as chaperone (Michalak *et al.*, 1999). To mediate its C1q effects, calreticulin has been shown to interact with surface molecule CD91 and enhanced clearance of apoptotic cells through binding to C1q-opsonized apoptotic cells (Ogden *et al.*, 2001).

1.3.3 α2β1/Very late antigen (VLA)-2/CD49b/CD29

α2β1, also known as VLA or CD49b/CD29, exists as a heterodimer and is a type I transmembrane receptor (Zutter and Edelson, 2007). Belonging to a subset of β1 (CD29) integrins which bind collagens (α1β1, α2β1, α10β1 and α11β1), α2β1 was shown to bind to the collagen tail of C1q through its α2 I domain (Edelson *et al.*, 2006). α2β1 also binds MBL and SP-A which could be mediated through their collagen tails (Edelson *et al.*, 2006). α2β1 is expressed by B cells, mast cells, monocytes, neutrophils, NK cells and T cells (Zutter and Edelson, 2007).

1.3.4 CD35/Complement receptor 1 (CR1)

CD35, also known as CR1, is a 190- 280 kD type I transmembrane receptor and has a pI of 6.57 (Lu *et al.*, 2008). It has a long extracellular domain which consists of 30 short consensus repeats (SCRs) each having 60 to 70 amino acids. These SCRs are further grouped in 4 long homologous repeats (LHRs) (LHR-A, -B, -C and -D) consisting of 7 SCRs. C1q was shown to bind to LHR-D (SCR 22 - 28) and the last two SCR (SCR 29 and 30) of CD35 and the collagen tail is responsible for the interaction between CD35 and C1q (Klickstein *et al.*, 1997). In addition, CD35 also recognizes C3b, C4b (Klickstein *et al.*, 1997) and MBL (Ghiran *et al.*, 2000) The receptor is found on B cells, erythrocytes, monocytes, neutrophils (Fearon, 1980) and T cells (Yaskanin and Waxman, 1995).

1.3.5 CD91/α2 macroglobulin receptor/Low density lipoprotein-related protein (LRP)

CD91, also known as α2 macroglobulin receptor or LRP, is a 500 kD type I transmembrane receptor and binds to at least 30 different ligands (Herz and Strickland, 2001). The extracellular domain is acidic having a pI of 5.16 (Lu *et al.*, 2008) which is further grouped into 4 ligand-binding clusters

(Cluster I - IV) (Herz and Strickland, 2001). Besides associating with calreticulin and help in the phagocytosis of C1q-opsonized apoptotic cells (Ogden *et al.*, 2001), C1q was shown to bind to CD91directly (Duus *et al.*, 2010). Both the globular head and collagen tail of C1q are involved in the binding to CD91 (Duus *et al.*, 2010). Although the exact binding site on CD91 is not known, it is suggested that C1q can bind to Cluster II, Cluster IV or both based on competitive binding with known CD91 ligands α2 macroglobulin and exotoxin A which binds to Cluster II and Cluster IV respectively (Duus *et al.*, 2010). The receptor is present on monocytes (Duus *et al.*, 2010), fibroblasts, hepatocytes and keratinocytes (Binder *et al.*, 2000).

1.3.6 CD93/C1q receptor for phagocytosis (C1qRp)

CD93, also known as C1qRp, is a 126 kD type I transmembrane receptor and has a C-type carbohydrate recognition domain and 5 EGF-like domains in its extracellular domain which has a pI of 4.95 (Lu et al., 2008; Nepomuceno et al., 1997). It is first identified as a C1q receptor by cloning based on its ability to enhance C1q-mediated phagocytosis of apoptotic cells (Nepomuceno *et al.*, 1997). The receptor recognizes the collagen tail of C1q and it also recognizes MBL and SP-A as all three molecules have similar structures (Nepomuceno *et al.*, 1997). However, the involvement of CD93 in enhancing C1q-mediated apoptotic cell clearance is unclear as $CD93^{-/-}$ macrophages showed similar enhancement in C1q-mediated phagocytosis (Norsworthy *et al.*, 2004). CD93 is expressed on B cells, endothelial cells, platelets, T cells and phagocytes such as DCs, macrophages and neutrophils (Greenlee-Wacker *et al.*, 2012).

1.3.7 Other C1q receptors

Beside the known C1q receptors such as calreticulin, gC1qR, α2β1, CD35, CD91 and CD93, recent studies have identified additional receptors that

interact with C1q and mediate its various functions (Ma *et al.*, 2012; Naito *et al.*, 2012; Son *et al.*, 2012).

Receptor for advanced glycation endproducts (RAGE) belongs to the Ig superfamily and is a multiligand receptor which binds to many proteins such as advanced glycation endproducts (AGE), high mobility group box 1 (HMGB1), S100 and collagen I (Sparvero *et al.*, 2009). It is recently shown to be a C1q receptor and helps in mediating adhesion of monocytes (Ma *et al.*, 2012). The receptor also enhanced phagocytosis of C1q-opsonized apoptotic cells by monocytes and can activate the complement system. An antibody recognizing C1q globular heads blocked C1q binding to RAGE whereas antibody against C1q collagen tails did not have any effect on C1q and RAGE interactions, indicating that C1q binds RAGE through its globular heads. Together with its co-receptor Mac-1, RAGE has higher affinity to C1q. RAGE is present on B cells, DCs, monocytes, macrophages, neutrophils and T cells (Sparvero *et al.*, 2009).

The Wnt pathways are involved in diverse biological functions and three different pathways can be activated upon Wnt receptor activation (Amin and Vincan, 2012; MacDonald and He, 2012). These pathways are the canonical Wnt/β-catenin pathway, the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway. The canonical pathway regulates transcription via β-catenin whereas non canonical pathways (PCP and Wnt/Ca^{2+} pathways) do not regulate the transcriptional activity of β-catenin directly. For the canonical pathway, Wnt receptors are heterodimers consisting of Frizzled (Fz), a G-coupled protein, and LRP5/6, a single transmembrane protein (Amin and Vincan, 2012; MacDonald and He, 2012). C1q is shown to bind to the cysteine-rich domain of Fz and activates the canonical Wnt signaling pathway (Naito *et al.*, 2012). Upon binding, C1q induced C1r/C1s activation which cleaved the extracellular domain of LRP5/6, leading to activation of Wnt signaling. Serum concentration of C1q increases with aging and subsequent Wnt signaling activation impairs muscle regeneration in aged mice (Naito *et al.*, 2012). Fz is widely expressed in most cells and these include DC, epidermal

cells, fibroblasts, satellite cells, stem cells and T cells (Huang and Klein, 2004; Naito *et al.*, 2012).

Lastly, C1q also binds to leukocyte-associated Ig-like receptor 1 (LAIR-1) through its collagen tail (Son *et al.*, 2012). This regulates the differentiation and activation of DCs. In addition, LAIR-2 was shown to bind to C1q though the interaction was weaker as compared to that of LAIR-1 (Son *et al.*, 2012). Similar to RAGE, LAIR-1 belongs to the Ig superfamily (Meyaard, 2008). It is a type I transmembrane receptor that has an extracellular C2-type Ig-like domain which binds to collagen and two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic tail. The main function of LAIR-1 is to inhibit the signals transduced by immunoreceptor tyrosine-based activating motifs (ITAMs)-bearing receptors but it can also inhibit cytokine-mediated signals. LAIR-1 is found on many immune cells including B cells, basophils, DCs, eosinophils, mast cells, monocytes, NK cells and T cells (Meyaard, 2008).

1.4 Producers of C1q

Most complement proteins are synthesized by hepatocytes in the liver. C1q is unique in which it is produced mainly by myeloid cells but not hepatocytes (Lu *et al.*, 2008). This is shown in a study where wild type mouse bone marrow cells are able to restore C1q production in irradiated $C1qA^{-/-}$ mice, suggesting C1q production by hemapoietic cells (Petry *et al.*, 2001).

1.4.1 Macrophages

Macrophages are derived from monocytes and they show a high degree of heterogeneity (Gordon and Taylor, 2005). Differences in macrophages reflect the specialized functions that they carry out in their respective tissues. For example, alveolar macrophages are responsible for clearing pathogens in the lungs, thymic macrophages and tingible-body macrophages help in the removal of apoptotic lymphocytes during the development of immune response in the germinal centre and osteoclasts are involved in bone remodeling (Gordon and Taylor, 2005).

Macrophages are one of the first few cell types known to produce C1q. Initial studies were mainly performed with peritoneal (Loos *et al.*, 1980; Loos *et al.*, 1981) and alveolar macrophages (Loos *et al.*, 1980) which showed the biosynthesis of C1q by macrophages. Subsequently, studies have also shown that other macrophages such as Kupffer cells (macrophages in the liver) (Armbrust *et al.*, 1997), microglial cells (macrophages in the brain) (Haga *et al.*, 1996; Schafer *et al.*, 2000) and bone marrow macrophages (Tripodo *et al.*, 2007) produce C1q.

1.4.2 DCs

Similarly, different subsets of DCs are found both in human and mouse (Shortman and Liu, 2002). The differences in the subsets are attributed to their

locations, migratory pathways, specific immunological function and the mechanism of generation. Despite the differences, all DC subsets are capable of antigen uptake, processing and presentation to naive T cells.

The presence of C1q in DC is first detected in follicular DCs and interdigitating DCs in the spleen of rat (Schwaeble *et al.*, 1995). Additional studies also showed the production of C1q by both mouse (Castellano *et al.*, 2010; Tripodo *et al.*, 2007) and human DCs (Cao *et al.*, 2003; Castellano *et al.*, 2004).

1.4.3 Other known C1q-producing cells

Other than macrophages and DC, many other cell types are shown to produce C1q. In the brain, besides microglial cells, retinal ganglion cells also expressed C1q and the expression is upregulated in the presence of astrocytes (Stevens *et al.*, 2007). During pregnancy, both DECs (Bulla *et al.*, 2008) and trophoblasts (Agostinis *et al.*, 2010) express C1q at the both the mRNA and protein levels. The expression of C1q is critical in trophoblast invasion and placental development (Agostinis *et al.*, 2010; Bulla *et al.*, 2008). Conflicting data exist with regards to whether monocytes produce C1q (Bensa *et al.*, 1983; Cao *et al.*, 2003; Tenner and Volkin, 1986). Lastly, chondrocytes (Bradley *et al.*, 1996), epithelial cells (Bing *et al.*, 1975; Colten *et al.*, 1968; Morris *et al.*, 1978), fibroblasts (Al-Adnani and McGee, 1976; Reid and Solomon, 1977) and mesenchymal cells (Morris *et al.*, 1978) are also known to produce C1q.

1.5 Aims of study

C1q is able to modulate diverse physiological and pathological functions (Lu *et al.*, 2008; Nayak *et al.*, 2010). These functions are not restricted to immune responses but can also be observed in development, pregnancy, aging and cancer. This is achieved by regulating the different cell types present in tissues and organs through binding multiple receptors with its globular heads and collagen tails and activating distinct signaling pathways.

Despite the fact that serum contains large amounts of C1q, it is still unknown if a large macromolecule like C1q with its extended structure is able to pass through the walls of blood vessels and access different tissues. This is especially so when blood vessels have limited permeability in the absence of inflammation. Thus, in order for C1q to exert its effects on the different cells types, local synthesis of C1q is important. This can be observed in arteries, articular cartilage, brain, esophagus, liver and placenta (Agostinis *et al.*, 2010; Armbrust *et al.*, 1997; Bobryshev *et al.*, 2010; Bradley *et al.*, 1996; Bulla *et al.*, 2008; Cao *et al.*, 2003; Schafer *et al.*, 2000; Stevens *et al.*, 2007). However, the possible cellular sources of C1q in many tissues and organs remain unknown.

Thus, the aim of the study is to identify cellular sources of C1q which can contribute C1q to their local microenvironments. In addition, the potential roles that C1q may play in the local microenvironment will be investigated. Osteoclasts are our candidate cells as they share many similarities with macrophages and DCs, known producers of C1q, which will be discussed later.

Chapter 2 – Materials and Methods

2.1 Antibodies, buffers and media

2.1.1 Antibodies

Table 2.1 provides details of the antibodies used throughout the study.

Table 2.1. List of antibodies used in the study.

Target	Host	Conjugation	Clone (Isotype)	Usage	Source
Primary Antibodies					
β-actin	Mouse	-	AC15 (IgG1)	WB	S-A
C1q	Goat	-	-	E, WB	S-A
C1q	Mouse	-	3R9/2 (IgG1)	E, FC, IF	AbD
CD1a	Mouse	PE	CB-T6 (IgG1)	FC	Ancell
CD11b	Mouse	PE	ICRF44 (IgG1)	FC	eBio
CD11c	Mouse	PE	B-ly6 (IgG1)	FC	BD
CD14	Mouse	PE	UCHM1 (IgG2a)	FC	Ancell
CD35	Mouse	-	E11 (IgG1)	FC	SC
CD35	Mouse	-	J3D3 (IgG1)	FC	SC
CD40	Mouse	PE	5C3 (IgG1)	FC	eBio
CD80	Mouse	PE	L307.4 (IgG1)	FC	BD

CD86	Mouse	PE	BU63 (IgG1)	FC	Ancell
CD91	Mouse	-	A2MR-α2 (IgG1)	FC	BD
CD93 (C1qRP)	Mouse	-	MAB4314 (IgG2b)	FC	Chemi
EEA-1	Rabbit	-		IF	SC
gC1q-R/p33	Mouse	-	MAB1160 (IgG1)	FC	Chemi
gC1q-R/p34	Mouse	-	MAB1161 (IgG1)	FC	Chemi
MHCI	Mouse	PE	3F10 (IgG2A)	FC	Ancell
MHCII	Mouse	PE	TDR31.1 (IgG1)	FC	Ancell
Secondary Antibodies					
Mouse IgG	Goat	FITC	-	IF	JI
Mouse IgG	Goat	AP	-	WB	B-R
Mouse IgG	Goat	HRP	-	E	Dako
Mouse IgG	Goat	PE	-	FC	Dako
Rabbit IgG	Goat	cy3	-	IF	JI
Goat IgG	Rabbit	AP	-	WB	B-R

Keys:

Usage

E – ELISA FC – Flow cytometry

IF – Immunofluorsence WB – Western blot

Source

AbD – AbD Serotec, Oxford; UK B-R – Bio-Rad, Hercules, CA

Chemi – Chemicon, Billercia, MA eBio – eBioscience, San Diego, CA

JI – Jackson Immunoreseach, West Grove, PA

S-A – Sigma-Aldrich, St Louis, IL

SC – Santa Cruz Biotechnology Inc, Dallas, TX

2.1.2 Buffers

2.1.2.1 Cell biology

1 x PBS pH 7.4 (Diluted from 10 x stock)

KH_2PO_4	1.76 mM
Na_2HPO_4	10.4 mM
NaCl	137 mM
KCl	2.7 mM

FACS wash buffer

1 x PBS	
Heat inactivated (HI) fetal bovine serum (FBS)	2.5% (v/v)
Sodium azide	0.05% (w/v)

2.1.2.2 Molecular biology

1 x TAE pH 8.0 (Diluted from 10 x stock)

Tris-acetate	40 mM
EDTA	1 mM

2.1.2.3 Protein chemistry

Tris buffered saline (TBS)

Tris-HCl pH 7.4	50 mM
NaCl	150 mM

10 x SDS-PAGE electrophoresis buffer

Tris base	250 mM
Glycine	2.5M
SDS	1% (w/v)

Adjust the pH to 8.3

5 x Reducing Laemmli buffer

Tris-HCl pH 6.8	250 mM
Glycerol	50% (v/v)
SDS	10% (w/v)
Bromophenol Blue	1% (w/v)
Dithiothreitol (DTT)	0.5 M

10 x Western blot transfer buffer

Tris base	250 mM
Glycine	1.92 M

For 1 x Western blot buffer, 1 unit of the 10 x buffer was added to 7 units of deionized water and 2 units of 100% methanol.

PBS-T/TBS-T buffer

1X PBS/TBS

Tween-20	0.05% (v/v)

Western blot blocking buffer and antibody diluent

1 x TBS-T

Non-fat milk	5% (w/v)

Western blot stripping buffer

Tris-HCl pH 6.8	62.5 mM
SDS	2% (v/v)
β-metacaptoethanol	0.1 M

2.1.3 Cell culture media

The following reagents for making the media were purchased commercially: αMEM, high glucose DMEM with L-Glutamine, RPMI-1640 (Invitrogen, Carlsbad, CA), BCS, FBS (Hyclone, Waltham, MA), 200 mM L-glutamine, 100 x penicillin/streptomycin (10,000 U/ml or 10 mg/ml) (PAA Laboratories, Pasching, Austria) and 10 x trypsin-EDTA (Sigma-Aldrich).

Monocyte adhesion media

RPMI-1640	
BCS	5% (v/v)
Penicillin/streptomycin	100 U/ml

Osteoclast culture media

αMEM	
HI FBS	10% (v/v)
L-glutamine	2 mM
Penicillin/streptomycin	100 U/ml

DC/Macrophage culture media

RPMI-1640	
BCS	10% (v/v)
L-glutamine	2 mM
Penicillin/streptomycin	100 U/ml
Sodium pyruvate	1 mM
β-metacaptoethanol	0.0012% (v/v)

Cell stimulation media

RPMI-1640	
HI BCS	10% (v/v)
Penicillin/streptomycin	100 U/ml

Cell culture media

High glucose DMEM	
HI FBS	10% (v/v)
L-glutamine	2 mM
Penicillin/streptomycin	100 U/ml

2.2 Cell biology techniques

2.2.1 Isolation of monocytes from human buffy coats

Enriched peripheral blood leukocytes were derived from healthy donors in the form of buffy coat preparations (National University Centre, Blood Donation Centre, Singapore). The buffy coat was diluted two-fold in PBS. 30 ml of the diluted buffy coat was layered over 12 ml of Ficoll-Paque (Amersham Bioscience Corp., Piscataway, NJ) in a 50 ml tube. The tube was centrifuged at 400 x g for 30 minutes (acceleration – 1, deceleration – 0). Peripheral blood mononuclear cells (PBMC) were collected at the gradient interface, PBS was added to 50 ml and the cells were centrifuged at 200 x g for 15 minutes. The cell pellet was resuspended and washed with PBS at the same conditions. Another two washes were performed at 100 x g for 10 minutes. The extensive washings were done to remove the platelets. The cells were then resuspended in 60 ml monocyte adhesion media, divided into three T75 cell culture flasks and cultured for 1 hour in a humidified incubator with 5% CO_2 at 37^oC.

The non-adherent cells, which were mainly lymphocytes, were removed with four washes using the monocyte adhesion media. The adherent cells, which were mainly monocytes, were harvested by gentle scraping. The purity of the isolated monocytes were at least 90% based on the expression of CD14 (Figure 3.1).

2.2.2 In vitro generation of DC, macrophages and osteoclasts from monocytes

2.2.2.1 Osteoclast culture

Osteoclasts were differentiated from the isolated monocytes as described previously (Quinn *et al.*, 1998). Briefly, monocytes were cultured at a density of 0.5 x 10^6 cells/ml in osteoclast culture media supplemented with

20 ng/ml M-CSF (R & D Systems Inc., Minneapolis, MN) and 40 ng/ml RANKL (Peprotech, Rocky Hill, NJ). This was done in 6-well culture plates with 2 ml of cell suspension seeded into each well. Half of the media was replaced with fresh media containing cytokines every 3 days.

Most of the experiments were performed using Day 8 osteoclasts. For experiments involving different time points, cells were harvested at each time point as indicated.

2.2.2.2 Macrophage culture

Macrophages were differentiated from monocytes by culturing the cells at a density of 0.5 x 10^6 cells/ml in DC/Macrophage culture media supplemented with 20 ng/ml M-CSF (R& D Systems Inc.). This was done in 6-well culture plates with 2 ml of cell suspension seeded into each well. Half of the media was replaced with fresh media containing cytokines every other day. The cells were used at either Day 6 or Day 8.

2.2.2.3 DC culture

DC were differentiated from monocytes by culturing the cells at a density of 0.5 x 10^6 cells/ml in DC/Macrophage culture media supplemented with 20 ng/ml GM-CSF and 40 ng/ml IL-4 (R & D Systems Inc). This was done in 6-well culture plates with 2 ml of cell suspension seeded into each well. Half of the media was replaced with fresh media containing cytokines every other day. The cells were used at either Day 6 or Day 8.

All cell cultures were performed in a humidified incubator with 5% CO_2 at 37^oC.

2.2.3 Culturing of cell lines

The DC2.4 and N9 cell lines were a generous gift from Dr Wong Siew Heng and the remaining cell lines were obtained from the American Type Culture collection (ATCC) (Rockville, MD). The cell lines used (Table 2.2) were maintained in cell culture media and cultured in a humidified incubator at 37^{o}C in the presence of 5% CO_2.

The cell lines were sub-cultured either using trypsin/EDTA or gentle scrapping. For the trypsin/EDTA method, the culture medium was first removed and the cells were washed with 1 x PBS. 2 ml of 1 x trypsin/0.5 mM EDTA (Sigma-Aldrich) was then added and incubated for 1-10 minutes at 37^{o}C depending on the cell line. 8 ml of cell culture media was added and the cell suspension was centrifuged at 400 x g for 5 minutes. The cell pellet was resuspended in cell culture media and the cells were seeded at a desired density in new T-75 flasks. For the gentle scrapping method, the adherent cells were removed by gentle scrapping with a cell scrapper, centrifuged at 400 x g for 5 minutes and the cell pellet was resuspended in cell culture media. Cells were then seeded at a required density in new T-75 flasks.

For long term cryostorage, the cells were resuspended to a density of 0.5 - 1 x 10^7 cells/ml in cell culture media. DMSO was added to the cell suspension to a final concentration of 10%. The cell suspension was then aliquoted into cryogenic vials (Nalge Nunc International A/S, Roskilde, Denmark), frozen at -80^{o}C overnight and transferred to liquid nitrogen for long term storage.

Table 2.2. List of cell lines used for the study.

Name	Cell Type	Tissue	Sub-culture ratio	Cell removal
Human Cell Lines				
HEK293T	Epithelial cell	Embryonic kidney	1:6, every 2 to 3 days	Trysin/ EDTA
HeLa	Epithelial cell	Cervix	1:6, every 2 to 3 days	Trysin/ EDTA
HepG2	Epithelial cell	Liver	1:6, every 2 to 3 days	Trysin/ EDTA
MCF-7	Epithelial cell	Mammary gland	1:4, every 2 to 3 days	Trysin/ EDTA
Murine cell lines				
C2C12	Myoblast	Muscle	1:8, every 2 to 3 days	Trysin/ EDTA
DC2.4	DC	Bone marrow	1:6, every 2 to 3 days	Trysin/ EDTA
N9	Microglia	Embryonic brain	1:6, every 2 to 3 days	Trysin/ EDTA
J774	Macrophage	Ascites	1:6, every 2 to 3 days	Scrapping
NIH-3T3	Fibroblast	Embryo	1:8, every 2 to 3 days	Trysin/ EDTA
RAW264.7	Macrophage	Ascites	1:6, every 2 to 3 days	Scrapping

2.2.4 Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was performed using the acid phosphatase/leucocytes (TRAP) kit (Sigma-Aldrich) with some modifications. Cells cultured in tissue culture plates were fixed with acetone/citrate/formaldehyde solution for 30 seconds. After fixing, the cells were washed extensively with deionized water and incubated with acetone/naphthol AS-BI phosphoric acid-tartrate staining solution for 1 hour at 37^{o}C in the dark. The cells were then washed with

deionized water, air-dried and examined using the Olympus IX81 inverted microscope and the ImagePro Plus software (Olympus Co., Tokyo, Japan).

2.2.5 Bone resorption assay

Monocytes were seeded on BioCoat^{TM} Osteologic discs (BD Biosciences, San Diego, CA) and differentiated into osteoclasts according to the method described in 2.2.2.1. After 8 days of culture, the cells were removed with bleach (6% NaOCl, 5.2% NaCl) and washed extensively with deionized water. Macrophages were also cultured on the discs as a control. The resorption pits were observed using the Olympus IX81 inverted microscope and the ImagePro Plus software (Olympus Co.).

2.2.6 Cell stimulation assay

Differentiated cells were harvested at Day 8 by gently scrapping using a cell scrapper and washed twice. For washings and culture media for stimulation, the osteoclast culture media was used for osteoclasts whereas the cell stimulation media was used for macrophages and DC. The cells were seeded at a density of 0.5 x 10^6 cells/ml in 96-well plates. 100 µl of the cell suspension was seeded in each well and each stimulus was performed in triplicates. For extraction of RNA and flow cyotmetry analysis, 1 x 10^6 cells were seeded per condition in 2 ml of media in a 6-well plate. 100 ng/ml IFNγ (R& D Systems Inc.) and 500 ng/ml LPS (Sigma-Aldrich) were used to stimulate the cells for 48 hours.

2.2.7 Cell adhesion assay

To examine the adherent properties of the cells, 96-well culture plates were pre-coated with BSA or C1q. 30 or 50 µg/ml of solution was normally used for coating. Both BSA and C1q were purchased from Sigma-Aldrich. In

some experiments, the concentration of solution used for coating varied. The plates were coated overnight at 4^{o}C and washed twice with PBS before use. 0.5 x 10^{6} cells (200 µl) were then seeded into each well and each condition was performed in triplicates. Adherent cells were normally determined after 24 hours of incubation. The cells were washed twice with warm culture medium before 100 µl of 0.1% (w/v) crystal violet in 10% ethanol (v/v) in PBS was added to the wells and incubated for 10 minutes at 37^{o}C. Crystal violet staining solution was then removed and the wells were washed thrice with warm PBS. 50 µl of 2% SDS was added to release crystal violet from the adherent cells and absorbance was read at 570 nm.

To compare the effects of immobilized and soluble C1q, 50 µg/ml of C1q was used to coat the culture plates for immobilized C1q whereas C1q was added to the culture medium to a final concentration of 50 µg/ml for soluble C1q. The cells were then incubated for 24 hours before microscopic examination was performed to determine the morphologies of the cells. BSA was used as a control for both immobilized and soluble states.

To inhibit protein synthesis, cycloheximide (CHX) was used to pre-treat the cells for 30 minutes before the cells were seeded into the wells.

2.3 Molecular biology techniques

2.3.1 Total RNA isolation and purification

Total RNA was extracted using the Nucleospin RNA II (Clontech, Mountain View, CA) kit following manufacturer's protocol. Briefly, cells were lysed with 350 µl buffer RA1 and 3.5 µl β-mecapthoethanol and mixed vigorously. 350 µl 70% ethanol was then added to the cell lysate and vortex twice for 5 seconds each. The mixture was transferred to the RNA column and centrifuged for 30 seconds. Subsequently, 350 µl membrane desalting buffer was added and centrifuged for 1 minutes. 95 µl DNase reaction mixture was added incubated at room temperature for 15 minutes and the reaction was stopped with 200 µl buffer RA2. The column was centrifuged for 30 seconds, washed twice with 600 µl and 250 µl buffer RA3 and centrifuging it for 30 seconds and 2 minutes respectively. Lastly, the RNA was eluted from the column with 40 µl of nuclease-free water. All centrifugations were performed at 11,000 x g. RNA concentration was determined using the Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA) and the A260/280 ratio was typically above 2.1. The isolated RNA was stored in -80^{o}C for long-term storage.

2.3.2 Reverse transcription

First strand cDNA was generated by reverse transcription (RT) from RNA using iScript^{TM} cDNA synthesis kit (Bio-Rad). 0.2 - 1.0 µg of RNA was normally used, added to 4 µl of 5 x iScript reaction mix and 1 µl of iScript reverse transcriptase and nuclease-free water was added to top up the reaction mix to 20 µl. The complete reaction mix was then incubated at 25^{o}C for 5 minutes, 42^{o}C for 30 minutes and 85^{o}C for 5 minutes using 2720 Thermal Cycler (Applied Biosystems Life Technologies, Carlsbad, CA). cDNA was diluted to 100 µl total volume with nuclease-free water and stored in -20^{o}C for long term storage.

2.3.3 Polymerase chain reaction (PCR)

PCR reactions were performed in a 25 µl reaction mix with 1 µl cDNA, 5 µl 5 x green reaction buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTP, 0.2 µl GoTaq DNA polymerase (Promega, Madison, WI), 1 µl forward and reverse primer mix (10 µM each) and 15.8 µl nuclease-free water. The conditions used were 95^{o}C for 2 minutes (initial denaturation), 35 cycles of 95^{o}C for 1 minutes, 5^{8o}C for 1 minute and 72^{o}C for 1 minute and 72^{o}C for 5 minutes (final extension). The PCR products were visualized on 1.5% (w/v) agarose gel.

The primers were designed using the PerlPrimer open source software (Marshall, 2004) or obtained from PrimerBank (Spandidos *et al.*, 2010; Wang *et al.*, 2012), a public resource with validated primers for PCR specificity and efficiency (Table 2.3). For the designing for primers, primers spanning two consecutive exons would be selected whenever possible so as to eliminate the possibility of amplifying the target gene on unspliced genomic DNA.

2.3.4 Quantitative real-time PCR (qPCR)

qPCR was performed in triplicates for each sample in a total reaction volume of 20 µl in a 96-well reaction plate (Applied Biosystems Life Technologies). Each reaction mix consisted of 2 µl cDNA, 10 µl 2 x GoTaq qPCR Master Mix (Promega), 1 µl forward and reverse primer mix (10 µM each) and 7 µl nuclease-free water. The reaction plate was then ran on the ABI Systems 7500 Real-Time PCR machine (Applied Biosystems Life Technologies) using the Comparative Ct quantitation method. The conditions for the run were 50^{o}C for 2 minutes, 95^{o}C for 10 minutes and 40 cycles of denaturation (95^{o}C for 15 seconds) and annealing and extension (60^{o}C for 1 minute). The dissociation curve analysis was performed at the end of each run to ensure the specificity of the primers used. Relative expressions of respective genes were calculated based on the ΔΔCt method by using GAPDH as the endogenous control. The primers used are listed in Table 2.3.

Table 2.3. List of primers used for PCR and qPCR.

Target gene	Forward (Sense) primer/ Reverse (Antisense) primer
Human Primers	
ACTB	ACCACACCTTCTACAATGA AAACATGATCTGGGTCATCTT
ACP5	TGCAGACTTCATCCTGTC CAAAGGTCTCCTGGAACC
C1QA	CTTCCTCATCTTCCCATCT GTTCAGCAGACACAGACA
C1QB	AGGCGTCTGACACAGTATG CCTGGAAGCCCTTTCTCT
C1QBP	ATCAACTCCCAATTTCGTGGTT TCCTCTGGATAATGACAGTCCAA
C1QC	ACCTGCAGTTCCTTCTCC TTCTCCCTTCTGCCCTTT
CA2	AAACACAACGGACCTGAG TTGCTTGATCATAGGAAACAG
CALCR	CTTCTTCTAAATCACCCAACC CATCCATCATCTTCTTTCGTC
CD93	CCGGAAGTAACATTGAGGGCT TCTGAGTCTCGTCCTTGTCAC
CR1	CACGAAGCCGCCAATTTGTC CCCACTTGATCGTCATTGCTG
CSF1	AGGAACAGTTGAAAGATCCA AGACATTCTTGACCTTCTCC
CSF1R	ACATTCATCAACGGCTCT CCTCATCACACCTATCAGT
CTSK	ATAACAACAAGGTGGATGAAA TGGGATATAAAGGGTGTCAT
GAPDH	CGGAGTCAACGGATTTGGTCG TCTCGCTCCTGGAAGATGGTGAT

IL6	CCCAGGAGAAGATTCCAA CTCTTGTTACATGTCTCCTTTC
IL10	AATGCCTTTAATAAGCTCCAAGA TCTCAGTTTCGTATCTTCATTGT
ITGA2	CCTACAATGTTGGTCTCCCAGA AGTAACCAGTTGCCTTTTGGATT
ITGB1	GTAACCAACCGTAGCAAAGGA TCCCCTGATCTTAATCGCAAAAC
LRP1	CTATCGACGCCCCTAAGACTT CATCGCTGGGCCTTACTCT
MMP9	CGGACCAAGGATACAGTT CAGTGAAGCGGTACATAGG
NFATC1	AAGAAGATGGTCCTGTCTG ACCAGAGAATTCGGCTTG
TGFB1	AACCCACAACGAAATCTATGA AATTGTTGCTGTATTTCTGGTA
TNFRSF11A	AGATCGCTCCTCCATGTA TGTACTTTCCTGGTTCACAT

2.4 Protein chemistry techniques

2.4.1 Preparation of cell lysate

Cells were harvested and washed thrice with PBS. Cold lysis buffer (Biosource, Camarillo, CA) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich) was added to the cells and incubated on ice for at 30 minutes. The cell lysates were vortexed occasionally to ensure complete lysis. After lysis, the cell lysates were centrifuged at maximum speed for 10 minutes to remove insoluble material. This was performed at 4^{o}C. The supernatant was then transferred to a clean tube and store at -80^{o}C until further use.

2.4.2 Determination of protein concentration

The protein concentrations of cell lysates were determined using the Bio-Rad protein assay kit. Eight serially diluted BSA standards were first prepared with the initial concentration of 1 mg/ml. 10 µl of the standards and samples (diluted 5 times) were then added into the wells of a 96-well plate. The 5x dye reagent was diluted with deionized water and filtered through a 0.45 µm filter. Subsequently, 200 µl of the diluted dye reagent was added to each well and the mixtures were incubated at room temperature for 5 minutes before absorbance was read at 595 nm. The standard curve was constructed by plotting the BSA concentrations against the absorbance at 595 nm and samples concentrations were determined from the curve.

2.4.3 SDS-PAGE

10 - 20 µg of protein samples were used for each condition and 5 x reducing Laemmli buffer was added to each sample. The samples were then incubated at 95^{o}C for 10 minutes to denature and reduce the proteins. After denaturation and reduction, the samples were centrifuged at maximum speed for 3 minutes before loading into the wells of a 10 or 12.5% SDS-PAGE gel

(Table 2.4). The proteins were resolved in 1 x SDS-PAGE electrophoresis buffer at 100 – 120 V until the samples reached the bottom of the gel.

Table 2.4. Composition of SDS-PAGE gel

Components	**Resolving gel**		**Stacking gel**
	10%	12.5%	4%
Water (ml)	4.02	3.22	3.02
0.5 M Tris-HCl pH 6.8 (ml)	-	-	1.25
1.5 M Tris-HCl pH 8.8 (ml)	2.5	2.5	-
10% (w/v) SDS (µl)	100	100	50
30% (w/v) Acrylamide/Bis (ml)	3.33	4.13	0.65
10% (w/v) APS (µl)	50	50	25
TEMED (µl)	5	5	5
Total volume (ml)	10	10	5

2.4.4 Coomassie Blue (Blue Silver) staining

To visualize the proteins in the SDS-PAGE gels, the gels were stained overnight using the Blue Silver staining protocol (Candiano *et al.*, 2004). The staining solution contained 10% (v/v) phosphoric acid, 10% (w/v) ammonium sulfate, 0.12% Coomassie G-250 (Bio-Rad) and 20% methanol dissolved in deionized water. Gels were destained with deionized water.

2.4.5 Western blotting

After SDS-PAGE, the proteins were electroblotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) with the 1 x Western blot transfer buffer at 100 V for 90 minutes at 4^{o}C. The membrane was then blocked with blocking buffer at room temperature for 1 hour. The membranes were washed before diluted primary antibody was added to the membrane and incubated at

4°C overnight. Washing was carried out followed by the addition of diluted secondary antibody. The membrane was incubated for 1 hour at room temperature and washing was performed after incubation. Proteins were visualized using the Immno-Star™ AP Chemiluminescent substrate (Bio-Rad). Primary and secondary antibodies were diluted in blocking buffer and each washing after incubation with antibodies consisted of washing thrice with TBS-T for 10 minutes each time. All incubations were performed on a shaker with constant shaking.

To detect another protein after chemiluminescent detection, the membrane was rinsed thrice with TBS-T. Next, 10 ml of Western blot stripping buffer was added to the membrane and sealed in ziplock bags. The membrane was then incubated at 50°C for 20 minutes with periodic shaking. After stripping, the membrane was washed thrice with TBS-T for 10 minutes each time, blocked and reprobed with another antibody according to the method mentioned previously. To ensure proper removal of the antibodies, the stripped membrane was incubated with chemiluminescent substrate and tested for the presence of any signal. The absence of signal indicates successful removal of the antibodies from the membrane.

2.4.6 Flow cytometry

2.4.6.1 Surface staining

Cells were harvested, washed twice with cold PBS and resuspended in cold 1% goat serum in PBS. Antibodies were then added to the cells and incubated on ice for 30 min. For flurochrome-conjugated antibodies, the cells were washed thrice with FACS wash buffer and fixed with cold 1% (w/v) paraformaldehyde (PFA) in PBS. For non-conjugated antibodies, the cells were washed twice and incubated with PE-conjugated goat anti-mouse antibody on ice for 30 minutes. Subsequently, the cells were washed thrice with FACS wash buffer, fixed with cold 1% PFA in PBS and stored in 4°C before analysis.

2.4.6.2 Intracellular staining

Cells were harvested and washed twice with PBS. The cells were then processed with the BD Cytofix/Cytoperm^{TM} Fixation/Permeabilization kit (BD Biosciences) according to the manufacturer's protocol. Briefly, 1 x 10^6 cells were fixed with 250 µl Fixation/Permeabilization solution on ice for 20 min and washed twice with Perm/Wash solution. The fixed and permeabilized cells were resuspended in 50 µl Perm/Wash solution containing 1% goat serum, incubated with antibody on ice for 30 – 60 min and washed thrice with Perm/Wash solution. PE-conjugated goat anti-mouse antibody was then added to the cells, incubated on ice for 30 – 60 min and washed thrice. The cells were resuspended in PBS containing 1% HI FBS and stored in 4^{o}C before analysis.

Flow cytometry analysis was carried out on the Dako CyAn flow cytometer using the Summit 4.3 software (Dako, Glostrup, Denmark).

2.4.7 Enzyme-linked immunosorbent assay (ELISA)

2.4.7.1 C1q ELISA

To detect C1q in cell culture supernatant, a lab-developed sandwich ELISA system was used. The capture antibody was polyclonal goat anti-C1q and 100 µl of the diluted (0.5 µg/ml diluted in PBS) was used to coat each well of the Maxisorp plates at 4^{o}C overnight. The wells were then washed, blocked with 3% BSA in PBS for 1 hour at room temperature and washed again. Samples or C1q standards were diluted in 1% BSA in PBS with 100 µl of sample/standard added into each well, incubated at 4^{o}C overnight and the wells were washed. 100 µl of monoclonal mouse anti-C1q antibody (0.4 µg/ml diluted in 1% BSA in PBS) was the added to each well, incubated at room temperature for 2 hours and washed. A goat anti-mouse IgG conjugated with HRP (Dako) was diluted in 1% BSA in PBS to a final concentration of 1 µg/ml and 100 µl was added to each well and incubated at room temperature for 2 hours in the dark. The wells were washed before 100 µl of TMB substrate was

added. The reaction was stopped with 50 µl of 1 M sulphuric acid and the absorbance was measured at 450 nm. All washes consisted of washing thrice with PBS-T.

2.4.7.2 Anti-C1q ELISA

To detect anti-C1q autoantibodies in plasma samples of SLE patients, a lab-developed ELISA system was used. 50 µl of C1q (5 µg/ml diluted in PBS) was used to coat each well of the Maxisorp plates at 4^{o}C overnight. The wells were then washed, blocked with PBS-T for 2 hours at room temperature and washed again. Samples were diluted in PBS-T containing 1 M NaCl with 50 µl of sample added into each well, incubated at 4^{o}C overnight and the wells were washed. 50 µl of monoclonal goat anti-human IgG antibody (0.5 µg/ml diluted in PBS-T containing 1 M NaCl) was the added to each well, incubated at room temperature for 2 hours and washed. A donkey anti-goat IgG conjugated with HRP (Dako) was diluted in PBS-T containing 1 M NaCl to a final concentration of 1 µg/ml and 50 µl was added to each well and incubated at room temperature for 2 hours in the dark. The wells were washed before 50 µl of TMB substrate was added. The reaction was stopped with 25 µl of 1 M sulphuric acid and the absorbance was measured at 450 nm. All washes before and after sample incubation were done with PBS-T and PBS-T containing 1 M NaCl respectively.

2.4.8 Immunofluorescence staining

Osteoclasts and macrophages were differentiated from monocytes on glass coverslips. Day 8 cells were washed thrice with PBS, fixed with 4% PFA in PBS for 20 minutes and washed 5 times with PBS. The cells were then permeabilized with permeabilizing buffer 0.1% (w/v) saponin in PBS for 30 minutes. Primary antibody, diluted in permeabilizing buffer containing 1% goat serum, was added and incubated for 1- 2 hours. Excess antibody was removed by washing the coverslips thrice with permeabilizing buffer. Next,

secondary fluorochrome-conjugated antibodies, diluted in permeabilizing buffer containing 1% goat serum, was added and incubated for 1 hour in the dark. For the staining of F-actin rings, rhodamine-phalloidin was added in this step. The coverlips were washed thrice with permeabilizing buffer and mounted on glass slide using VectaShield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA). All incubations were performed at room temperature.

For the stimulation of osteoclasts, the coverlips were incubated with the stimulants for 48 hours before immunofluorescence staining was carried out. The stained cells were then examined using the Olympus BX-60 digital microscope/ImagePro Plus software or Leica TCS SP5 confocal microscope/LeicaAF software. Co-localization between two signals were determined by Pearson's correlation analysis which was performed using the LeicaAF software.

2.4.9 Immunohistochemistry

Immunohistochemistry was performed by Dr Yuri V. Bobryshev, Faculty of Medicine, University of New South Wales.

Tissue specimens of femoral bone were obtained at the Department of Pathological Anatomy, 1st Medical Institute, Leningrad/St Petersburg, Russia. The study was carried out in accordance with the principles outlined in the Helsinki Declaration of 1975, as revised in 1983. Tissue specimens were fixed in 10% neutral-buffered formalin solution and were decalcified for 5 h using Decalcifying Solution-Lite (Sigma-Aldrich) prior to further processing and embedding into paraffin blocks. These blocks were cross-cut into 3 μm tissue sections. For single immunostaining, after elimination of endogenous peroxidase activity by 3% H_2O_2, sections were pre-incubated with normal non-immune serum and then tested by avidin-biotin complex (ABC) using a standard ABC immunoperoxidase method as described previously(Bobryshev *et al.*, 2010; Cao *et al.*, 2003). C1q was identified using a goat anti-C1q

antibody (Sigma-Aldrich). After washing in Tris-PBS (TPBS), pH 7.6, the sections were incubated with a biotin-labelled secondary antibody, followed by a treatment with ABC. After washing in TPBS, brown staining was produced by 5 min treatment with 3,3'-diaminobenzidine (DAB). All of the incubations were completed at room temperature (22◦C). For negative controls, a non-immune goat IgG was used in place of the anti-C1q antibody. None of the negative control sections showed positive immune staining. Counterstaining was performed with Mayer's haematoxylin.

2.4.10 IgG Precipitation

IgG precipitation was carried out to isolate the anti-C1q autoantibodies from SLE patients. 10 patients with the highest amount of anti-C1q autoantibodies were selected and 100 µl of sample was used for IgG precipitation. The samples were pooled and warmed to 25^{o}C. 0.18 g sodium sulphate was added into the serum and vortexed to ensure the salt was completely dissolved. The solution was allowed to precipitate for 30 minutes at 25^{o}C and the precipitate was pelleted at 3,000 g for 30 minutes at 25^{o}C. 500 µl of water was used to dissolve the pellet. Subsequently, 0.044g sodium sulphate was dissolved in the solution and allowed to precipitate for 30 minutes at 25^{o}C. The pellet was dissolved in 500 µl of water and stored in 4^{o}C till future uses.

2.4.11 Preparation of different C1q domains

2.4.11.1 Preparation of C1q collagen tails

C1q was first dialyzed with 100 mM sodium acetate pH 4.5 at 4^{o}C overnight. Subsequently, 5% (w/w) pepsin (Sigma-Aldrich) was added and incubated at 37^{o}C for 24 hours. The digestion was stopped by adjusting the pH of the mixture to 7.0 using 1 M Tris. C1q collagen tails were purified by using the ÄKTA FPLC machine with Mono S^{TM} 5/50 GL column (GE Healthcare, Piscataway, NJ). 100 mM sodium acetate pH 5.0 containing 150 mM NaCl

was used to equilibrate the column and bounded C1q collagen tails were eluted with 100 mM sodium acetate pH 5.0 containing 1M NaCl on a 50% salt gradient. Isolated C1q collagen tails were then dialyzed with 1X PBS. The purity of the collagen tails was determined by Coomassie blue staining and its concentration was determined by densitometry using intact C1q as standards.

2.4.11.2 Preparation of C1q globular heads

C1q was first dialyzed with 25 mM Tris pH 7.4 and 10 mM $CaCl_2$ at 4^oC overnight. Subsequently, 2.5 units collagenase, Type III (Sigma-Aldrich) was added for every 10 μg of C1q used and incubated at 37^oC for 16 hours. C1q globular heads were purified by using the ÄKTA FPLC machine with Mono S™ 5/50 GL column (GE Healthcare). 25 mM Tris pH 7.4 containing 10 mM $CaCl_2$ and 150 mM NaCl was used to equilibrate the column and bounded C1q globular heads were eluted with 25 mM Tris pH 7.4 containing 10 mM $CaCl_2$ and 1M NaCl on a 50% salt gradient. Isolated C1q globular heads were then dialyzed with 1X PBS. The purity of the collagen tails was determined by Coomassie blue staining and its concentration was determined by densitometry using intact C1q as standards.

2.5 Experimental repeats and statistical analysis

Most experiments were performed three times and the figures presented are representative of these experiments unless stated otherwise. Data were expressed as mean values of experimental triplicates ± standard error. Student's t test (two-sided, unpaired) was used to determine statistical significance unless stated otherwise. For significance level in the graphs, * respresents $p < 0.05$, ** respresents $p < 0.01$ and *** represents $p < 0.001$.

Chapter 3 – C1q production by osteoclasts and its regulation of osteoclastogenesis

3.1 Introduction

Despite serum having abundant C1q, tissue access to C1q may be limited in the absence of inflammation. Thus, local synthesis of C1q is critical in mediating the diverse functions of C1q. Unlike most of the other complement proteins which are synthesized by the hepatocytes, C1q is mainly produced by myeloid cells – macrophages and DCs (Lu *et al.*, 2008). Present in most tissues, they are APCs which are key scavengers and are involved in the induction and maintenance of tolerance, providing a link between the innate and adaptive immune system (Guermonprez *et al.*, 2002; Martinez-Pomares and Gordon, 2007; Mellman and Steinman, 2001; Unanue, 1984).

Macrophages display a high degree of heterogeneity in our bodies with various forms in different organs (Gordon and Taylor, 2005). This raises the question if the production of C1q is a common characteristic of these cells. Studies have shown that Kupffer cells and microglial cells express C1q (Armbrust *et al.*, 1997; Haga *et al.*, 1996; Schafer *et al.*, 2000). However, the production of C1q by osteoclasts – macrophages in the bone – has not been determined previously. Osteoclasts are chosen for several reasons. Firstly, osteoclasts share the same precursor cells as macrophages and DCs (Boyle *et al.*, 2003) – the main producers of C1q in the body (Lu *et al.*, 2008). Secondly, osteoclasts are demonstrated to be immune-competent and can act as APCs (Grassi *et al.*, 2011; Kiesel *et al.*, 2009; Li *et al.*, 2010). Lastly, as the plasma concentration of C1q is approximately 180 mg/L (Sontheimer *et al.*, 2005), the bones which constitute a large part of our body may be a likely source of serum C1q. Thus, we investigate the possibility of C1q production by osteoclasts and the likely role that C1q may play in the bone microenvironment.

3.2 Generation and characterization of in vitro differentiated osteoclasts

3.2.1 Differentiation of monocytes into osteoclasts and characterization of osteoclasts

For this part of the study, primary osteoclasts, macrophages and DCs are differentiated from human monocytes for all experiments. Monocytes were isolated from buffy coats of healthy blood donors using Ficoll-Paque gradient centrifugation followed by plastic adhesion methods. Each buffy coat typically yielded 10 - 40 million monocytes. The purity of the monocytes was determined by flow cytometry using CD14, a marker for monocytes, and at least 90% of the cells expressed CD14.

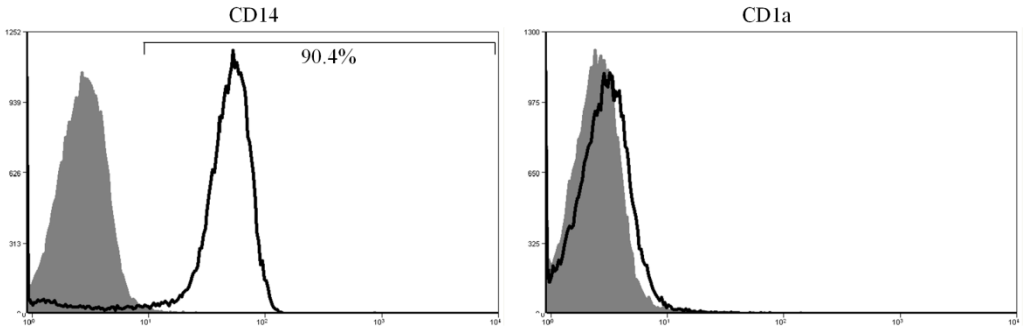


Figure 3.1. Surface expression of CD14 on isolated human monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat using the Ficoll-Paque gradient centrifugation method and platelets were removed by washing repeatedly. The cells were then resuspended and allowed to adhere onto plastic culture flasks for 1 h. Non-adherent cells were removed by multiple washings and the remaining adherent cells were harvested with gentle scraping. These cells were then stained with antibodies against CD14 and CD1a to determine the purity of the isolated cells. Solid histogram represents isotype control and black line represents CD14 or C1a.

Osteoclasts were then differentiated from the isolated monocytes based on the method described by Quinn *et al.* in which M-CSF and RANKL were added were added to the culture and incubated for 8 days (Quinn *et al.*, 1998). This was done in 6-well culture plates and 1 million monocytes were seeded per well. Half of the media was replaced with fresh media containing cytokines

every 3 days. The Day 8 cells were subjected to a series of experiment for osteoclast characterization.

Firstly, by microscopic examination, osteoclasts were observed to be much larger than macrophages (Figure 3.2A). In addition, osteoclasts were heterogeneous in shape and size whereas macrophages displayed uniformity in their shape and size (Figure 3.2A). Next, the expression of different osteoclast markers [calcitonin receptor (CTR) (*CALCR*), carbonic anhydrase II (CAII) (*CA2*), Cathepsin K (CatK) (*CTSK*), matrix metallopeptidase 9 (MMP9) (*MMP9*) and tartrate-resistant acid phosphatase (TRAP) (*ACP5*)] (Boyle *et al.*, 2003; Sorensen *et al.*, 2007) was examined using quantitative real-time PCR (qPCR). Osteoclasts expressed increased levels of these markers by 1.2 to 65 fold as compared to that of macrophages (Figure 3.2B). By TRAP staining, high level of the protein, as indicated by the purple granules, was also found in osteoclasts and these were largely absent in macrophages (Figure 3.2A). This is reflective of the increased expression of TRAP mRNA in osteoclasts as compared to macrophages. The majority of the osteoclasts was multinucleated as shown by DAPI staining whereas macrophages are mononucleated (Figure 3.2C). A small population of mononucleated cells was also found in the osteoclast culture and they are considered as osteoclast precursor cells or preosteoclasts. This is consistent with previous studies which showed heterogeneity in osteoclast cultures (Grassi *et al.*, 2003; Sorensen *et al.*, 2007).

In the bone, osteoclasts function as the main effector cells for bone resorption. To facilitate resorption, osteoclasts form a tight, sealed compartment – the resorption lacunae – against the bone surface which is mediated mainly by a ring of filamentous actin (F-actin) fibres and $\alpha_v\beta_3$ integrins (Nakamura *et al.*, 2007; Vaananen and Horton, 1995). This allows the acidification of the resorption lacunae and secretion of proteases for bone resorption to occur (Teitelbaum, 2000). F-actin rings were present in the multinucleated osteoclasts whereas no such structures were seen in the macrophages (Figure 3.2C). Lastly, the resorption ability of the *in vitro*-derived osteoclasts was determined. Monocytes were seeded and differentiated to osteoclasts in chamber slides coated with calcium phosphate to mimic the

bone surface. After 8 days, the cells were removed and resorption pits of various sizes were seen in the osteoclast culture (Figure 3.2D, right panel). However, no resorption pits were detected in the macrophage culture (Figure 3.2D, left panel). This is consistent with the upregulation of genes such as CA II, CatK and MMP9 (Figure 3.2B) which are all involved in the resorption process (Teitelbaum, 2000). These results showed that the *in vitro* generated osteoclasts are similar to the *in vivo* osteoclasts in terms of morphological features and functionality.

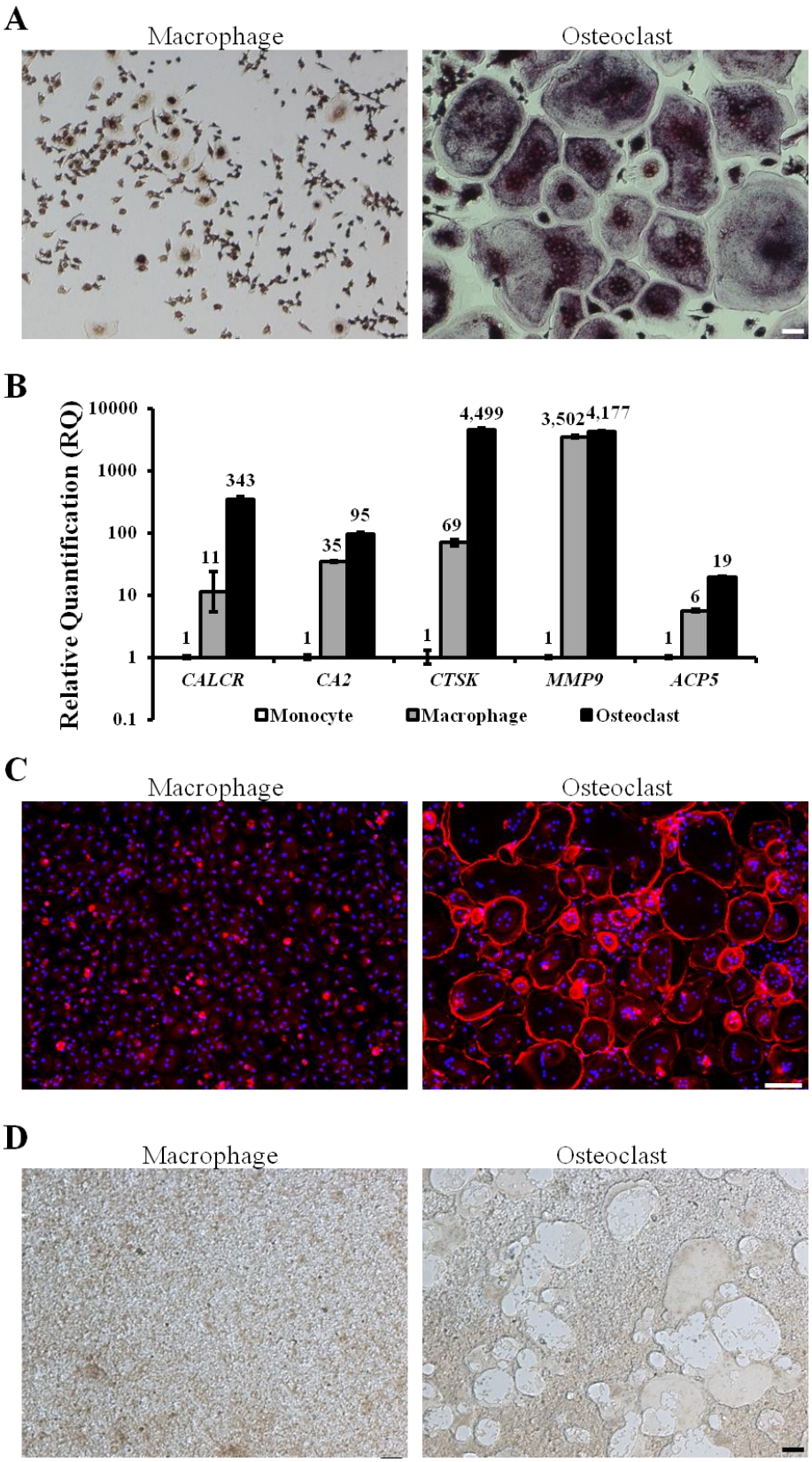


Figure 3.2. Generation and characterization of osteoclasts. Isolated monocytes were cultured in αMEM supplemented with M-CSF and RANKL for differentiation into osteoclasts. As a control, macrophages were differentiated from monocytes by only adding M-CSF. The cells were cultured

for 8 days with half of the medium replaced with fresh medium containing cytokines every 3 days. **(A)** Macrophages (left panel) and osteoclasts (right panel) grown in tissue-culture plates were fixed and stained to detect the expression of (tartrate-resistant acid phosphatase) (TRAP) (osteoclast marker) using the Acid Phosphatase, Leukocyte (TRAP) kit. The presence of TRAP will cause the formation of purple granules in the cytoplasm of the cells. **(B)** Quantitative real-time PCR (qPCR) was carried out to detect the expression of osteoclast markers using cDNA - reverse-transcription (RT) from total RNA - of monocytes, macrophages and osteoclasts. The expression levels of these markers for macrophages and osteoclasts represent the fold increase as compared to that of monocytes which were normalized to 1. *GAPDH* was used as the endogenous control. The data is representative of three independent experiments. **(C)** Macrophages (left panel) and osteoclasts (right panel) were grown on coverslips for 8 days. After differentiation, the cells were fixed, permeabilized and stained with DAPI and rhodamine-phallodin to observe the nuclei and characteristic F-actin rings (red) of osteoclasts respectively. **(D)** Macrophages (left panel) and osteoclasts (right panel) were differentiated on BD BioCoat^{TM} Osteologic^{TM} Discs to assess the resorption ability of the *in vitro* generated osteoclasts. Scale bar represents 25 μm.

3.2.2 Phenotype of *in vitro* osteoclasts

Recently, osteoclasts were demonstrated to be immune-competent and can act as APCs (Grassi *et al.*, 2011; Kiesel *et al.*, 2009; Li *et al.*, 2010). Thus, the surface expression of immune molecules which are important in antigen presentation was determined. For comparison, known APCs such as macrophages and DCs were used as controls. Phenotypic analysis showed that all three cell types expressed MHC molecules, MHC class I and MHC class II, and costimulatory molecules such CD80, CD86 and CD40. In addition, these cells also expressed CD11b and CD11c. CD1a, a DC marker, was only found on the surface of DC and not on the surfaces of osteoclasts and macrophages (Figure 3.3). However, CD14 was prominently expressed by both osteoclasts and macrophages and not by DC (Figure 2). The results showed that osteoclasts expressed surface molecules which are necessary for antigen presentation and are consistent with previous reports (Grassi *et al.*, 2011; Kiesel *et al.*, 2009). Thus, the above results showed that the *in vitro* generated osteoclasts not only have the traditional characteristics of osteoclasts (expression of osteoclast markers, formation of F-actin rings and resorption) but they also possess immunogenic properties discovered recently.

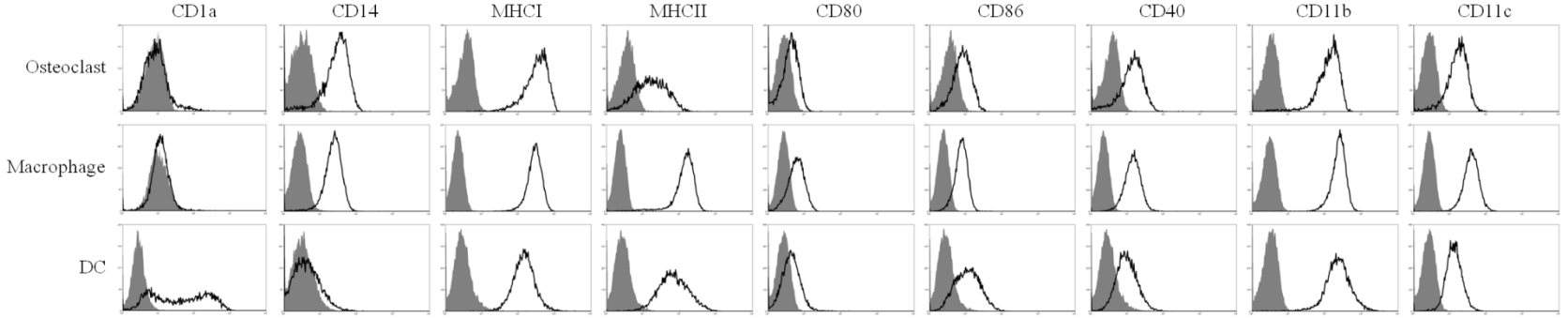


Figure 3.3. Phenotypic properties of osteoclasts, macrophages and dendritic cells (DCs). Monocytes were differentiated into osteoclasts (top row), macrophages (middle row and DC (bottom row) after 8 days of culture using M-CSF + RANKL, M-CSF and GM-CSF + IL-4 respectively. The cells were harvested and stained with flurochrome-conjugated antibodies against CD1a, CD14, MHCI, MHCII, CD80, CD86, CD40, CD11b and CD11c. Surface expression of these molecules was determined by flow cytometry. The results shown are representative of three independent experiments. Solid histogram represents isotype control and black line represents the respective antibodies.

3.3 Production of C1q by *in vitro* osteoclasts

3.3.1 Expression of *C1Q* mRNA by *in vitro* osteoclasts

With the generation of *in vitro* osteoclasts, the possibility of C1q production by these cells was first determined at the mRNA levels. This was done by qPCR and cells were observed for 21 days of culture. For comparison, the expression of *C1QA*, *C1QB* and *C1QC* in macrophages and DCs was also examined. Monocytes, which produce insignificant amounts of C1q (Bobryshev *et al.*, 2010; Cao *et al.*, 2003), were used as a negative control. All three *C1Q* subunits were detectable in developing osteoclasts by Day 3 and their expressions peaked at Day 6 which remained unchanged for the rest of the culture period (Figure 3.4). However, the expression of *C1Q* in osteoclasts was lower than that of macrophages and DC. Macrophages had the highest expression of *C1Q*, followed by DCs and then osteoclasts (Figure 3.4). Very low levels of *C1Q* mRNA were detected in monocytes by qPCR and RT-PCR (Figure 3.4, Appendix 1). CatK, an osteoclast marker, was used as a control. As shown in Figure 3.4, osteoclasts had the highest expression of *CTSK* as compared to macrophages and DCs which had very low levels of expression. This validates the osteoclastogenic property of the cells and the results show that osteoclasts, like macrophages and DCs, obtain increased *C1Q* mRNA expression as they differentiate from monocytes.

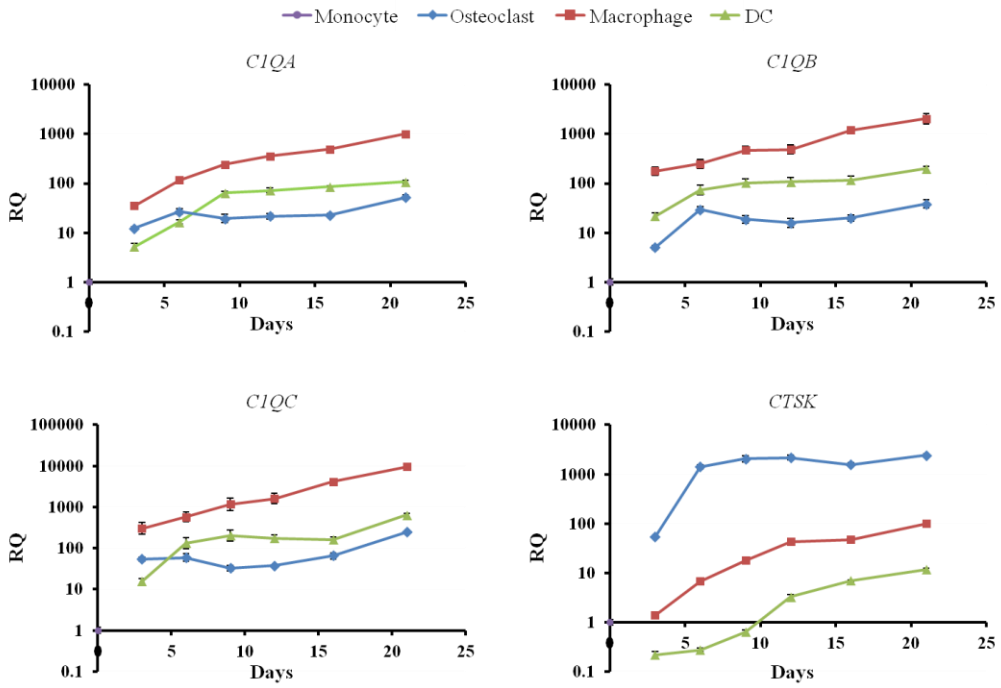


Figure 3.4. C1q expression by monocytes, osteoclasts, macrophages and DCs at the transcriptional level. Osteoclasts (◆), macrophages (■) and DCs (▲) were differentiated from monocytes for different time points (3, 6, 9, 12, 16 and 21 days) and total RNA was isolated at each time point. The RNA of monocytes (●) was also isolated. cDNA was generated by RT and qPCR was used to detect the expression levels of *C1QA*, *C1QB* and *C1QC* mRNA in these cells. The expression levels of *C1QA*, *C1QB* and *C1QC* for osteoclasts, macrophages and DCs represent the fold increase as compared to that of monocytes which were normalized to 1. Cathepsin (CatK) (*CTSK*) and *GAPDH* were used as osteoclast marker and endogenous control respectively. The graphs are representative of three independent experiments.

3.3.2 Production and secretion of C1q by *in vitro* osteoclasts

Given that the cultured osteoclasts expressed *C1Q* mRNA, the production of C1q at the protein level was evaluated. Similarly, cells were harvested over 21 days at various time points and the cells lysates were subjected to Western blotting. A polyclonal anti-C1q antibody was used to detect the presence of C1q and a 26-27 kD band, equivalent to the sizes of C1qA and C1qB chains, was found in the osteoclast culture across various time points (Figure 3.5). A smaller and fainter band of approximately 24kD, equivalent to the size of C1qC chain, was also found in some of the samples. Osteoclasts produced detectable amounts of C1q protein by Day 3, peaked at

Day 6 and had approximately the same amount of C1q protein for the rest of the culture period (Figure 3.5A) and this is reflective of the *C1Q* mRNA expression in osteoclasts (Figure 3.4). C1q protein was not detected in monocytes (Figure 3.5A) which produce insignificant amounts of C1q as reported in previous studies (Bobryshev *et al.*, 2010; Cao *et al.*, 2003). This is in contrast with the very low level of RNA detected in monocytes as shown in Figure 3.4 and Appendix 1.

Consistent with the *C1Q* mRNA results (Figure 3.4), in general, macrophages and DCs produce more C1q as compared to osteoclasts (Figure 3.5A). Macrophages have a similar pattern of expression as osteoclasts in which C1q protein was detected by Day 3, peaked at Day 6 and maintained its expression for the rest of the culture period. Interestingly, C1q production in DCs was only detected on Day 6 whereas C1q protein was found in both macrophages and osteoclasts on Day 3 (Figure 3.5A). This resulted in the delay of its peak production of C1q (Figure 3.5A). The reason for the overall delay may be due to the low *C1Q* mRNA expression levels in DC at Day 3 (Figure 3.4).

As C1q is a plasma protein, the secretion of C1q by osteoclasts, macrophages and DCs was examined using sandwich ELISA. Culture media were collected over the 21 days of culture and used for analysis. As shown in Figure 3.5B, secreted C1q was generally undetectable for all three cell types on Day 3 despite the presence of intracellular C1q in osteoclasts and macrophages (Figure 3.5A). Similar to C1q mRNA and protein expression (Figures 3.4 and 3.5A), macrophages secreted the highest amount of C1q whereas osteoclasts secreted the least amount of C1q. In addition, macrophages and DCs showed increasing levels of secreted C1q over the culture period, indicating the continual secretion by these cells. However, C1q secretion by osteoclasts seemed to plateau in the later stages of the culture period which is reflective of the constant expression and production of C1q after Day 6 of culture (Figures 3.4 and 3.5A).

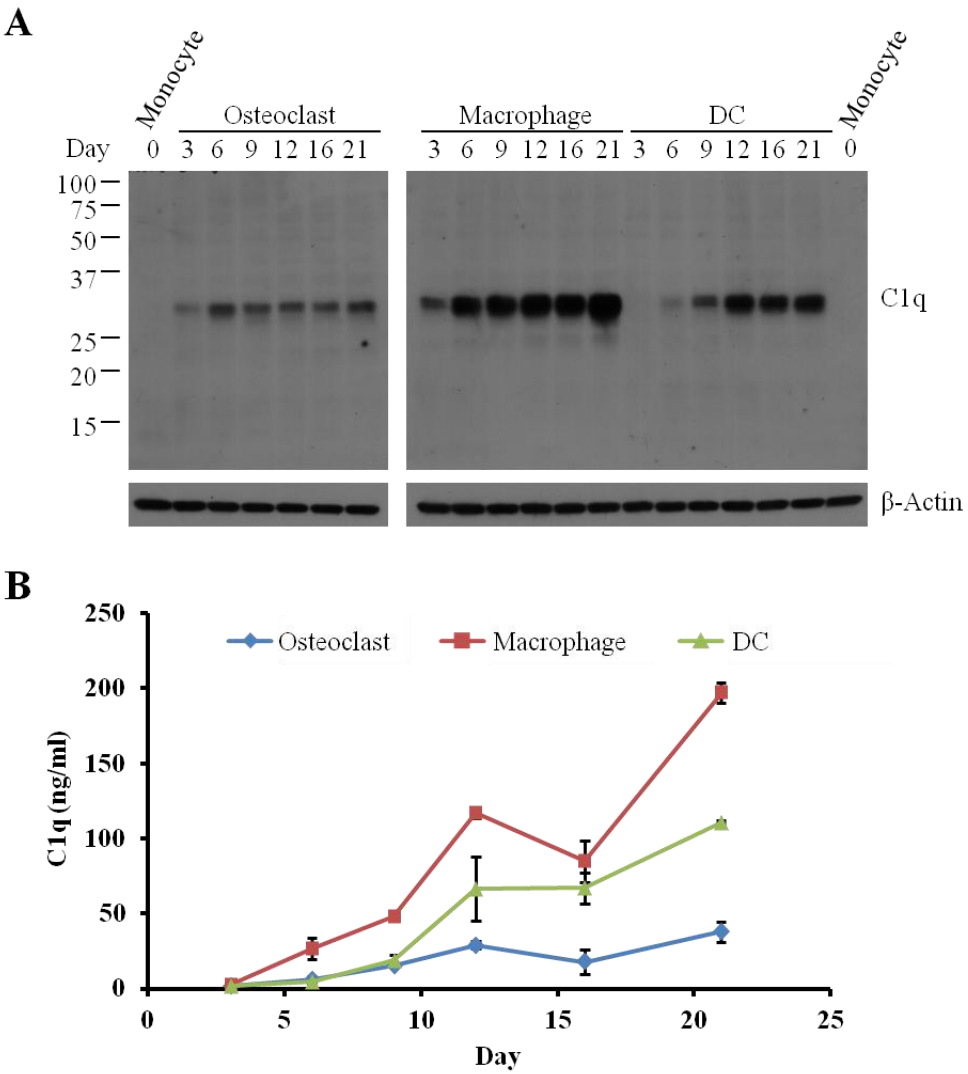


Figure 3.5. Production and secretion of C1q by osteoclasts, macrophages and DCs. Osteoclasts, macrophages and DCs were differentiated for different time points (3, 6, 9, 12, 16 and 21 days). The cells were lysed and media were collected at each time point. **(A)** Cell lysates of monocytes, osteoclasts, macrophages and DCs were processed for Western blotting and probed for the presence of intracellular C1q. β-actin was used as an endogenous control. **(B)** The amounts of C1q secreted by osteoclasts (◆), macrophages (■) and DCs (▲) into the media at different time points were determined by ELISA. The results shown are representative of three independent experiments.

3.3.3 C1q is preferentially produced by preosteoclasts

Given that cultured osteoclasts is heterogeneous (Figures 3.2A and 3.2C), consisting of both preosteoclasts and mature osteoclasts representing mono- and multinucleated osteoclasts respectively, it is interesting to determine which population of cells produce C1q. Immunofluorescence

microscopy was used as it allows the study of C1q production at single cell level. As shown in Figure 3.6, C1q was detected in only a fraction of cells and these were mainly the preosteoclasts. No signal was detected in the mature osteoclasts indicating that the multinucleated cells produced low levels of C1q (Figure 3.6). However, not all mononucleated cells were stained for C1q and this may be due to the differences in the developmental stages for the cells. To determine if the intracellular C1q detected is synthesized *de novo*, early endosomes were also co-stained using an antibody against the early endosomal marker, early endosome antigen 1 (EEA-1). This is to rule out that the possibility that C1q was taken up from extracellular medium via endocytosis. However, no co-localization was observed between C1q and EEA-1 stainings (Figure 3.6). In addition, by Pearson's correlation analysis, no significant co-localization was obtained for the C1q and EEA-1 signals (data not shown). Thus, this implies that the detected C1q molecules are synthesized *de novo* by the cultured osteoclasts.

Based on the results obtained so far, C1q is indeed produced and secreted by *in vitro* osteoclasts especially by the preosteoclasts. This may account for the constant C1q levels detected in the osteoclast culture media after 9 days of culture as there were more mature osteoclasts than preosteoclasts (Figure 3.5B). The preferential production of C1q by preosteoclasts may imply a role for C1q during the differentiation of monocytes to osteoclasts.

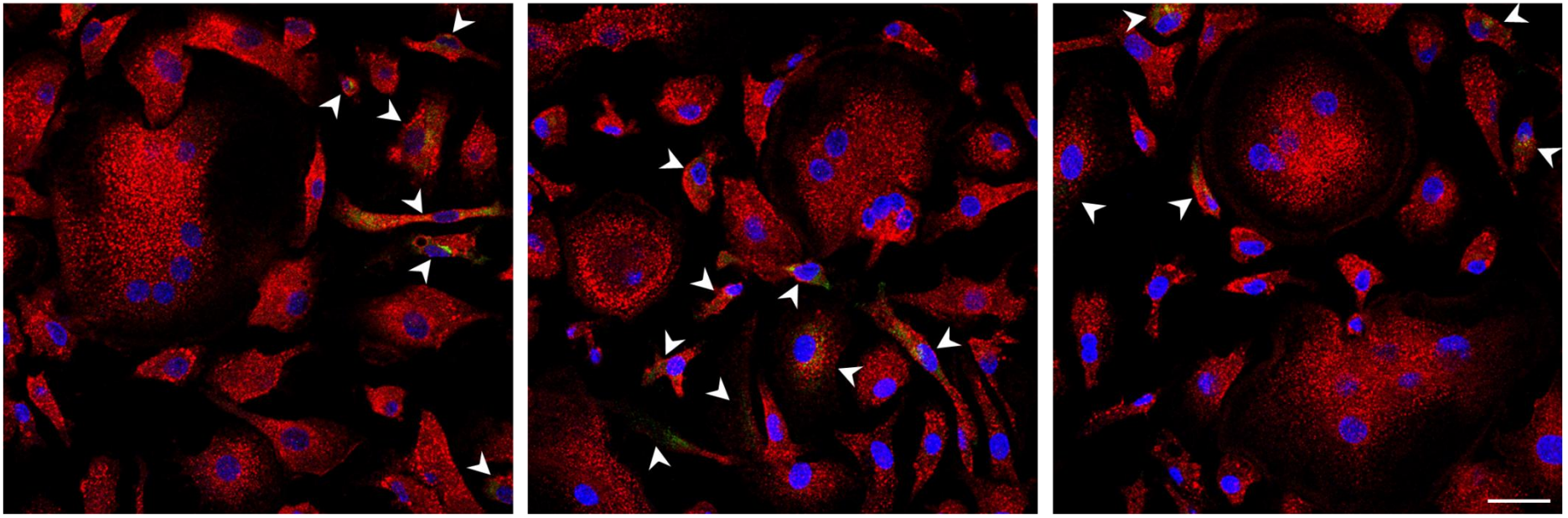


Figure 3.6. Preferential production of C1q by preosteoclasts. Osteoclasts were grown on coverslips and immunofluorescence staining was performed after 8 days of culture. Confocal immunofluorescence microscopy analysis was performed using anti-C1q (green) and anti-EEA-1 (red) antibodies. Arrowheads indicate C1q-positive mononucleated osteoclasts. Scale bar represents 25 μm. The results shown are representative of three independent experiments.

3.4 Induction of C1q in osteoclasts by IFNγ

3.4.1 Induction of C1Q gene expression by IFNγ and LPS

The production of C1q in macrophages can be regulated by many stimuli which include microbial structures, cytokines, hormones and drugs and IFNγ is one of them (Lu *et al.*, 2008). A recent study also showed that IFNγ induced C1Q gene expression in DCs (Chen *et al.*, 2011). However, the effects of IFNγ on C1q production in osteoclasts are unknown. Thus, Day 8 osteoclasts were stimulated with IFNγ for 48 hours and the cells were analyzed at RNA and protein levels. Lipopolysaccharide (LPS) was also used for the stimulation. Macrophages and DCs were also similarly treated and used for comparison. After stimulation with IFNγ, osteoclasts showed a significant increase in *C1Q* gene expression compared to the untreated cells (Figure 3.7A, left panel). LPS also increased the C1Q gene expression in osteoclasts though the effect was not as great as IFNγ (Figure 3.7A, left panel). Similar results were also observed for macrophages and DCs (Figure 3.7A, middle and right panels).

3.4.2 Increased synthesis and secretion of C1q by IFNγ-, but not LPS-, treated osteoclasts

Next, protein expression was investigated after the cells were stimulated with IFNγ and LPS and intracellular C1q was evaluated by flow cytometry. As seen in Figure 3.7B, untreated osteoclasts showed low level of intracellular C1q whereas both macrophages and DCs had significantly higher amounts of intracellular C1q. This is consistent with the Western blot result (Figure 3.5A). Upon stimulation with IFNγ, all three cell types displayed drastic increase in intracellular C1q and the effect was more pronounced in osteoclasts (Figure 3.7B). In contrast to the substantial upregulation of C1Q gene expression by LPS (Figure 3.7A), moderate increase was observed for the intracellular C1q levels in LPS treated cells as compared to the untreated cells (Figure 3.7B).

In addition, fluorescence microscopy was also used to determine the effects of IFNγ and LPS on the production of intracellular C1q in osteoclasts. Day 10 osteoclasts also the staining pattern as the Day 8 osteoclasts in which intracellular C1q could be detected mainly in the preosteoclasts (Figures 3.6 and 3.7C, left panel). Upon IFNγ stimulation, C1q was detected in both the preosteoclasts and mature osteoclasts (Figure 3.7C, middle panel). The signals in the IFNγ-stimulated cells were more intense as compared to the untreated cells, indicating the increased production of C1q by IFNγ (Figure 3.7C, left and middle panels). Similar to the untreated osteoclasts, the preosteoclasts still produced higher levels of C1q as compared to the mature osteoclasts in IFNγ-stimulated osteoclasts despite the general increased production of C1q (Figure 3.7C, left and middle panels). No significant increase in C1q staining was observed for LPS-stimulated osteoclasts (Figure 3.7C, right panel).

Lastly, the effects of IFNγ and LPS on secreted C1q were determined by ELISA. C1q secretion by untreated osteoclasts was low but this was increased substantially when IFNγ was used to stimulate the cells (Figure 3.7C, left panel). Untreated macrophages and DCs were able to secrete significant amounts of C1q and IFNγ further increased the C1q secretion by several folds (Figure 3.7C, middle and right panels). Once again, LPS had limited effects on C1q secretion for all three cell types (Figure 3.7C). Although the significant induction of C1q by IFNγ and the function of osteoclast-derived C1q remain to be investigated, these results consistently point to the production and secretion of C1q by osteoclasts.

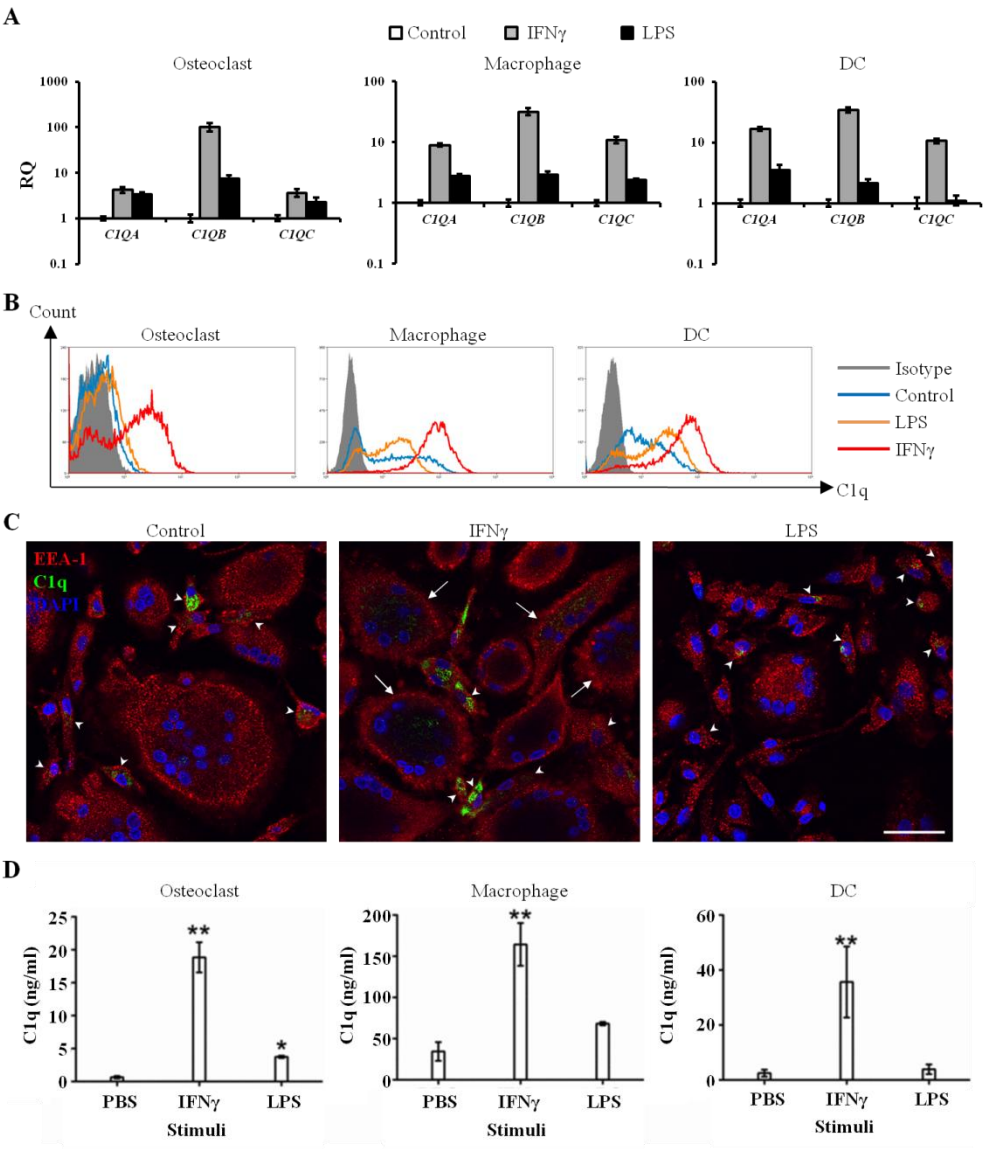


Figure 3.7. Induction of C1q production in osteoclasts, macrophages and DCs by IFNγ. Osteoclasts, macrophages and DCs were cultured for 8 days, washed and stimulated with IFNγ (100 ng/ml) or LPS (500 ng/ml) for 48 hours. **(A)** qPCR was performed to quantify the expression levels of *C1QA*, *C1QB* and *C1QC* after stimulation. For each cell type, the expression levels of *C1QA*, *C1QB* and *C1QC* for IFNγ- and LPS-treated cells represent the fold increase as compared to that of control cells which were normalized to 1. *GAPDH* was used as the endogenous control. **(B)** After stimulation, the cells were harvested, fixed, permeabilized and stained with an anti-C1q antibody. Flow cytometry analysis was carried out to determine the levels of intracellular C1q. Isotype control (—), control (—), IFNγ (—) and LPS (—). **(C)** Osteoclasts were grown on coverslips and treated with IFNγ (middle panel) and LPS (right panel) or left untreated (left panel) for 48 hours. Confocal immunofluorescence microscopy analysis was performed using anti-C1q (green) and anti-EEA-1 (red) antibodies. Arrows and arrowheads indicate C1q-positive multinucleated and mononucleated osteoclasts respectively. Scale bar represents 25 μm. The results shown are representative of three independent experiments. **(D)** ELISA was done to determine the amounts of secreted C1q by the cells after stimulation. Results are average for three independent experiments.

3.5 Presence of C1q in endogenous osteoclasts

To validate the production of C1q by *in vitro* generated osteoclasts, endogenous osteoclasts were examined for the production of C1q. Human femoral bone samples were fixed, decalcified and 3 μm sections were stained for the presence of C1q. Both endogenous mononucleated and multinucleated cells, representing preosteoclasts and mature osteoclasts, showed C1q staining near the bone tissues with the preosteoclasts having higher C1q staining as compared to that of mature osteoclasts (Figures 3.8 and 3.9). It was also noted that the mature osteoclasts displayed intense perinuclear staining (Figure 3.9). The results obtained are similar to the observations made for *in vitro* osteoclasts except that little C1q was detected in the *in vitro* mature osteoclasts (Figure 3.6). This may reflect the imperfect culture conditions for the *in vitro* osteoclasts. Collectively, the *in vitro* and *in vivo* results showed that osteoclasts are indeed C1q producers.

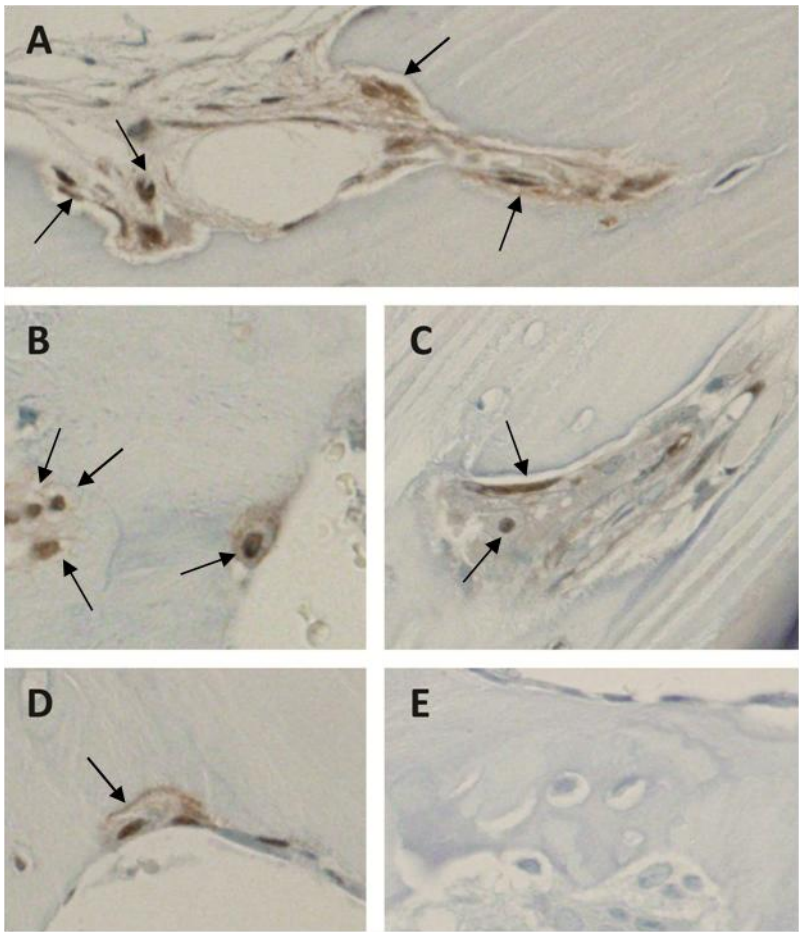


Figure 3.8. Presence of C1q-positive mononucleated cells in human femoral bone. 3 μm sections of human femoral bone samples were obtained and stained using anti-C1q or pre-immunization IgG (negative control) antibody. **(A-D)** The presence of C1q was indicated by brown staining which was produced using the standard avidin-biotin complex (ABC) immunoperoxidase method and 3,3'-diaminobenzidine (DAB) substrate. Counterstaining was done with Mayer's haematoxylin. **(E)** Negative control. Arrows indicate C1q-positive mononucleated cells. (Images are courtesy of Dr Yuri V. Bobryshev, Faculty of Medicine, University of New South Wales.)

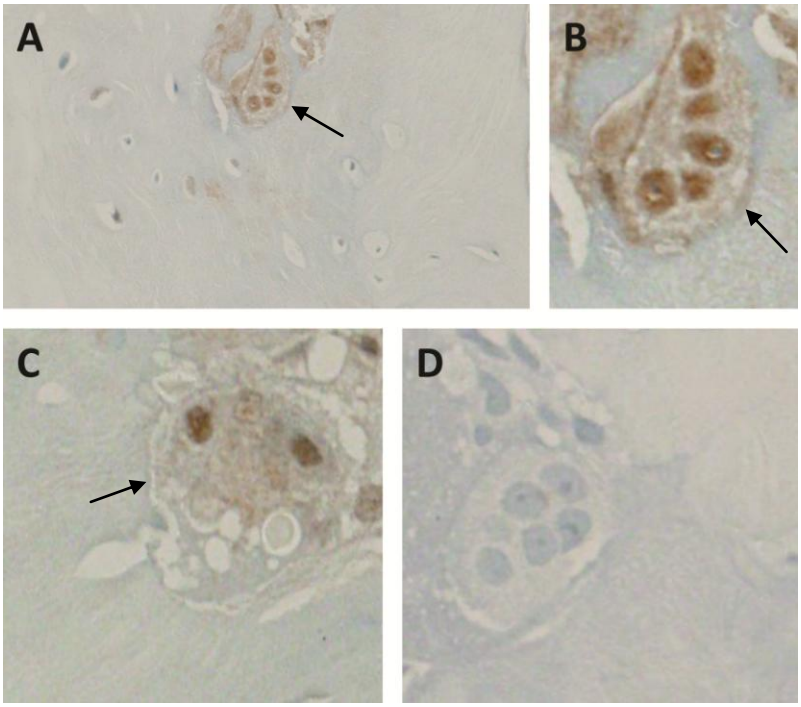


Figure 3.9. Presence of C1q-positive multinucleated cells in human femoral bone. 3 μm sections of human femoral bone samples were obtained and stained using anti-C1q or pre-immunization IgG (negative control) antibody. **(A-C)** The presence of C1q was indicated by brown staining which was produced using the standard ABC immunoperoxidase method and DAB substrate. Counterstaining was done with Mayer's haematoxylin. **(D)** Negative control. **B** is a magnification of **A**. Arrows indicate C1q-positive multinucleated cells. (Images are courtesy of Dr Yuri V. Bobryshev, Faculty of Medicine, University of New South Wales.)

3.6 Immobilized C1q augments osteoclastogenesis

Given that C1q was mainly found in preosteoclasts (Figure 3.6), we hypothesized that C1q may play a role in osteoclastogenesis – a process in which mature osteoclasts are formed from precursor cells (Boyle *et al.*, 2003; Teitelbaum and Ross, 2003). Previously, C1q was shown to regulate the differentiation of DC from monocytes (Castellano *et al.*, 2007; Teh *et al.*, 2011). One study shows that the addition of soluble C1q inhibited the responses of DC when stimulated with LPS (Castellano *et al.*, 2007). Another study shows that immobilized C1q caused DC to acquire tolerogenic phenotypes (Teh *et al.*, 2011). In addition, recent studies have shown that complement proteins are able to regulate osteoclastogenesis (Ignatius *et al.*, 2011; Sato *et al.*, 1993; Tu *et al.*, 2010). Thus, the effects of both soluble and immobilized C1q on the differentiation of monocytes to osteoclasts are examined. The cells were cultured for 8 days and bovine serum albumin (BSA) was used as a control for both soluble and immobilized C1q. Gene expression of osteoclast markers such as nuclear factor of activated T cells cytoplasmic 1 (NFATc1) (*NFATC1*), CTR (*CALCR*), CAII (*CA2*), CatK and TRAP (*ACP5*) were first examined. As shown in Figure 3.10, for osteoclasts cultured in the presence of immobilized C1q (imC1q osteoclasts), the expression of these osteoclast markers were upregulated 2 - 7 folds. In contrast, the markers of osteoclasts cultured with soluble C1q (sC1q osteoclasts) were not affected except for the slight induction in the expression of CTR which was not significant (Figure 3.10).

Subsequently, the effects of C1q on the expression of other molecules involved in osteoclastogenesis were determined. *CSF1R* [M-CSF receptor (M-CSFR, also known as c-fms)] and *TNFRSF11A* (RANK) were upregulated at least 2 folds in imC1q osteoclasts and this was not observed in sC1q osteoclasts (Figure 3.10). This may account for the increased expression of osteoclast markers in imC1q osteoclasts as these two receptors mediate the actions of M-CSF and RANK, the two essential cytokines required for osteoclastogenesis (Boyle *et al.*, 2003; Nakashima *et al.*, 2012; Teitelbaum and Ross, 2003).

The gene expression of cytokines such as M-CSF (*CSF1*), IL-6 (*IL6*), IL-10 (*IL10*) and TGF-β1 (*TGFB1*), which are produced by osteoclasts and regulate osteoclastogenesis (Boyle *et al.*, 2003; Evans and Fox, 2007; Jilka *et al.*, 1992; Kaneda *et al.*, 2000; Kurihara *et al.*, 1990; Mohamed *et al.*, 2007; Quinn *et al.*, 2001), were also examined. A drop in *CSF1* expression was observed for imC1q osteoclasts although the decrease was not statistically significant (Figure 3.10). *IL6* was upregulated in both imC1q and sC1q osteoclasts though the upregulation for sC1q osteoclasts was not significant (Figure 3.10). IL-6 is known to promote osteoclastogenesis (Jilka *et al.*, 1992; Kurihara *et al.*, 1990) and this may also cause the increased expression of osteoclast markers for imC1q osteoclasts. A significant increase in *IL10* expression was seen in imC1q osteoclasts (Figure 3.10). Despite IL-10 is shown to inhibit osteoclastogenesis (Evans and Fox, 2007; Mohamed *et al.*, 2007), the inhibition may not be effective in the presence of other osteoclastogenesis promoting factors. No difference was observed in the expression *TGFB1* for both types of C1q osteoclasts (Figure 3.10). Collectively, the results showed that immobilized C1q positively affects osteoclastogenesis. This could be due to the effective cross-linking of C1q receptor(s) by immobilized C1q as compared to soluble C1q or the exposure of conformation-dependent binding sites on immobilized C1q.

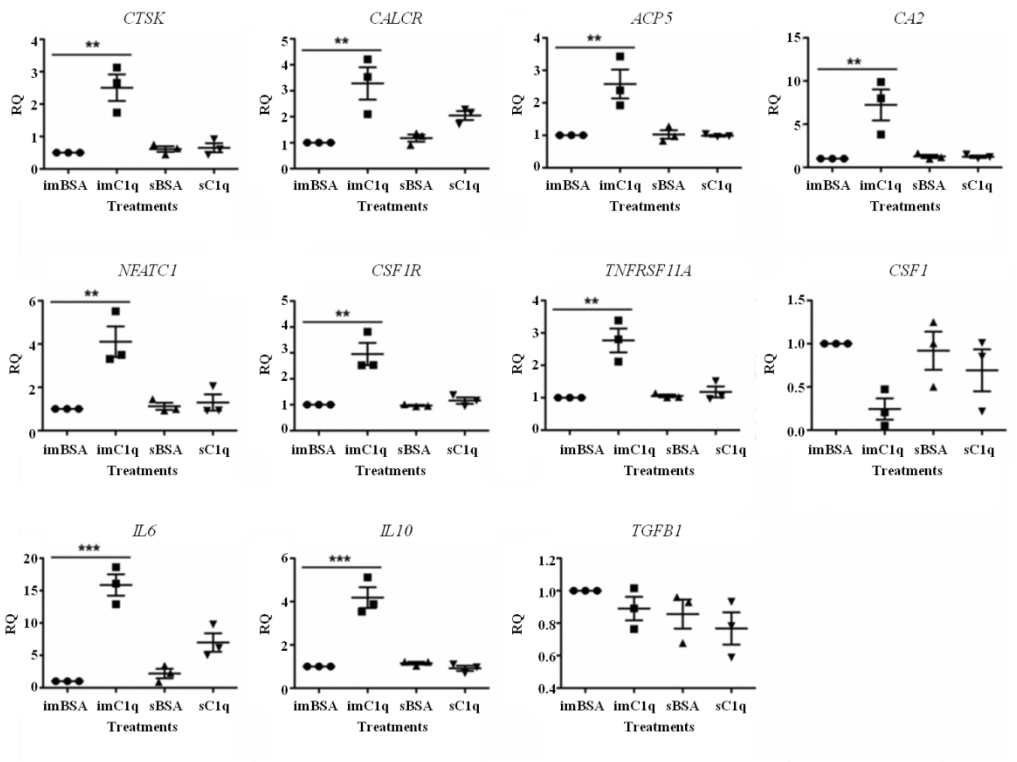


Figure 3.10. Induction of gene expression for osteoclast markers by immobilized C1q. Osteoclasts were differentiated from monocytes in the presence of immobilized BSA (imBSA), immobilized C1q (imC1q), soluble BSA (sBSA) (50 μg/ml) and soluble C1q (sC1q) (50 μg/ml) for 8 days. For imBSA and imC1q, 50 μg/ml of the proteins was used to coat the wells. Total RNA was then isolated and qPCR was carried out to determine the expression levels of different molecules. The expression levels of these molecules for osteoclasts generated in imC1q, sBSA and sC1q represent the fold increase as compared to that of osteoclasts generated in imBSA which were normalized to 1. GAPDH was used as the endogenous control. Results are average of three independent experiments. Statistical analysis was performed by one-way ANOVA and Bonferroni's post-test.

Chapter 4 – Anti-adhesive effects of solid phase C1q

4.1 Introduction

We have shown that osteoclasts produce C1q and preosteoclasts express more C1q as compared to that of mature osteoclasts. This leads us to investigate the effect of C1q on the development of osteoclasts. Immobilized C1q augments osteoclastogenesis and this is likely due to the upregulation of the receptors for M-CSF and RANKL, M-CSFR and RANK respectively, which renders the cells more sensitive to the cytokines used for differentiation.

Previously, our lab has also shown that immobilized C1q leads to the generation of tolerogenic DCs (Teh *et al.*, 2011). These DCs produced more anti-inflammatory cytokine IL-10 but lesser proinflammatory cytokines such as IL-12 and IL-23. In addition, imC1q DC displayed increased ERK, p38 and p70S6 kinase activation than control DC when stimulated with LPS. Lesser Th1 and Th17 induction from allogenic CD4 T cells was observed for these DC in mixed lymphocyte reaction.

These data indicate that immobilized C1q is able to interact with receptors present on monocytes and regulate their differentiation to DC and osteoclasts. We also observed that immobilized C1q induced distinct morphological changes in monocytes and they were less adherent (data not shown). DC cultured in the presence of immobilized C1q displayed similar expression of surface molecules as control DCs (Teh *et al.*, 2011). However, these DC still retained the distinct morphological changes observed in monocytes and were also less adherent. Hence, understanding how C1q affects cellular morphology will provide insights on the possible mechanisms involved in the regulation of monocyte development by C1q. These observations indicate that these two cell types may express a common receptor which mediates the effects of immobilized C1q.

Thus, we hypothesized that a common receptor is present on monocytes and DCs that interacts with immobilized C1q and affect the morphological and

adherent properties of these cells. The presence of such a receptor in other cell types which might mediate the effects of immobilized C1q will also be investigated. By identifying the receptor, this could provide more understanding on how immobilized C1q modulates monocyte development. This will provide more insights into the role C1q plays in maintaining tolerance and preventing diseases such as SLE.

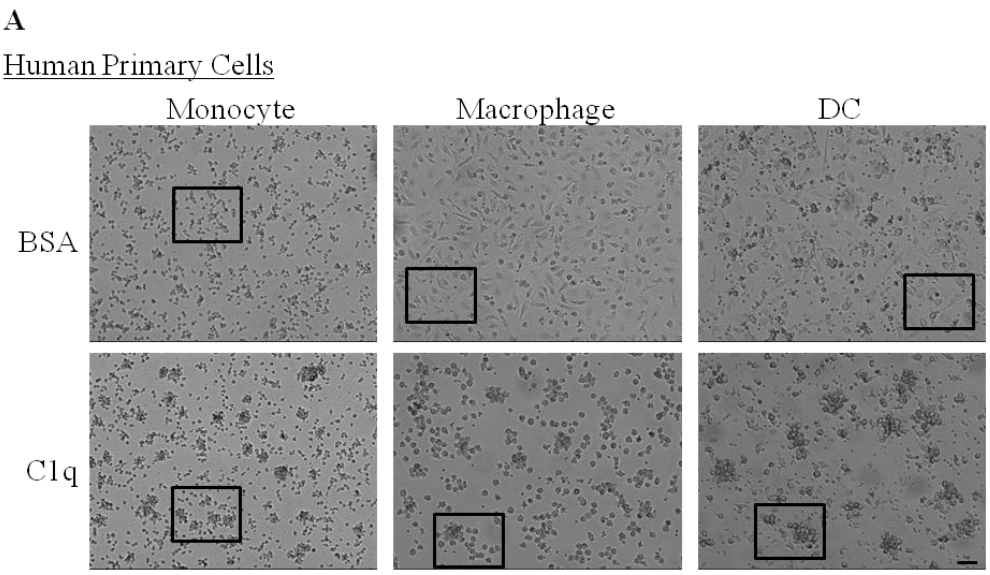
4.2 Immobilized C1q affects the morphologies and adherence of cells

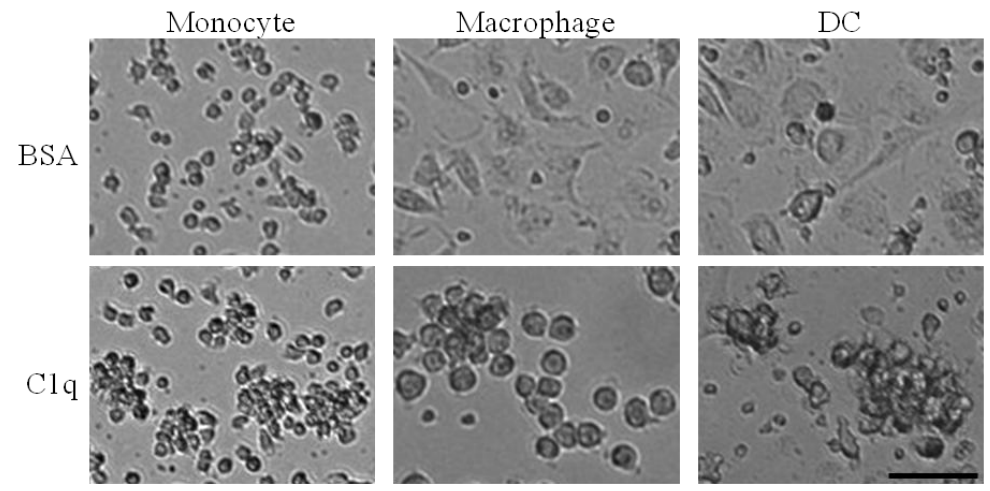
4.2.1 Immobilized C1q, but not soluble C1q, induces morphological changes in different cell types

To test the hypothesis, we first differentiated monocytes into macrophages and DCs. After 6 days of culture, the macrophages expressed high levels of CD14 whereas the DCs expressed high levels of C1a, indicating that the cells are fully differentiated (Figure 3.3). C1q was then immobilized on the culture plates before monocytes, macrophages and DCs were added. As a control, BSA was also used to coat the plates. The morphologies of the cells were subsequently observed under the microscope after 24 hours of culture. As seen in Figure 4.1A, cells cultured with immobilized C1q exhibited aggregation and were less spread out as compared to control cells. Upon closer examination, imC1q cells, especially macrophages and DC, appeared more rounded and tended to stick together (Figure 4.1B, lower panels). On the other hand, control macrophages and DCs appeared flatter and had more cellular extensions (Figure 4.1B, upper panels). For monocytes, clustering of the cells was apparent though spreading of the cells was not affected (Figure 4.1B). The insignificant changes in the spreading of monocytes are not surprising as monocytes are known to have little cytoplasmic extensions (Almeida *et al.*, 2001).

Based on the results, it seems that the effects of immobilized C1q are not limited to monocytes. This raises the question if other cell types would be similarly affected by immobilized C1q. Different cell lines were then used in the same assay to determine the effects of immobilized C1q on these cells. Similarly, immobilized C1q induced aggregation and rounding up of cells for both human and murine cell lines (Figures 4.1C and 4.1D). Cells cultured with immobilized BSA appeared normal and spread out nicely. For human cell lines, HEK293T cells displayed the most significant changes whereas the other cells lines had both rounded cell clusters and normal spreading cells (Figure 4.1C). For murine cell lines, the immune cell lines (DC2.4, J774, RAW264.7 and N9) showed distinct clustering of cells and the cells were more rounded (Figure

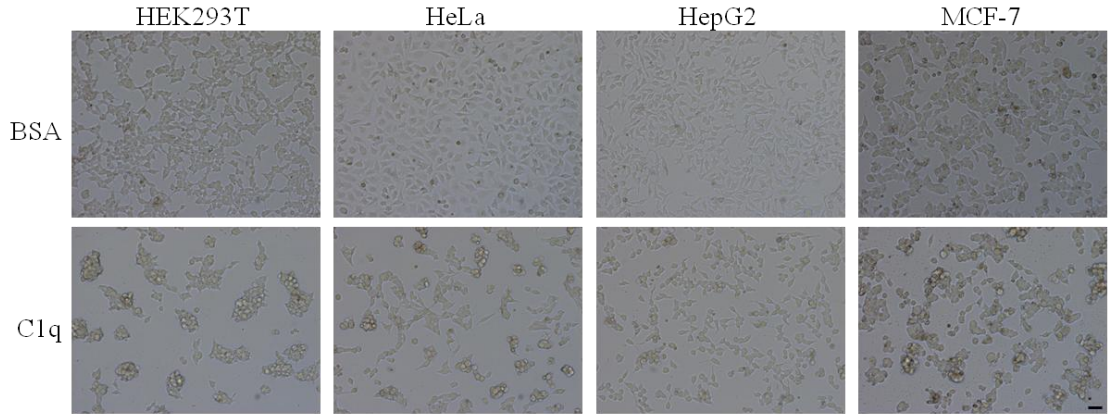
4.1D). The non-immune cell lines (C2C12 and NIH-3T3) were also affected by immobilized C1q in which cells were clustered and rounded though some cells remained extended (Figure 4.1D). Thus, immobilized C1q is able to affect the morphologies of different cell types.





C

Human Cell Lines



D

Murine Cell Lines

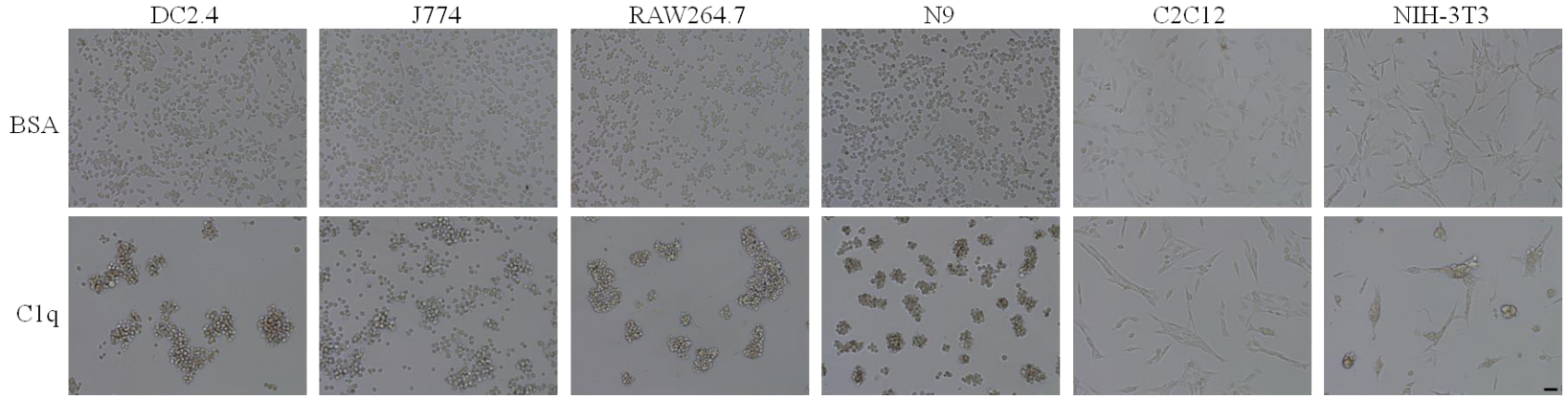


Figure 4.1. Morphological changes induced by immobilized C1q in different cell types. BSA and C1q were coated on tissue culture plates before cells were seeded into the individual wells. The cells were examined under the microscope after incubating for 24 hours. **(A)** Human primary cells including monocytes, macrophages and DCs were used for the experiment. **(B)** The boxed areas in **A** were enlarged to show changes in cell morphologies for cells in C1q-coated wells as compared to control cells. **(C)** Human cell lines were used and they were differentially affected by immobilized C1q. **(D)** Similarly, mouse cells lines were incubated with immobilized C1q and their morphologies were also affected. Scale bar represents 25 μm. The results are representative of three independent experiments.

Previously, in another study (Castellano *et al.*, 2007), soluble C1q has been shown to be able to generate DC with a rounded morphology. Thus, soluble C1q was also used to determine if it can mediate morphological changes in the cells. Using HEK293T and RAW264.7 cells, which exhibit the most obvious morphological changes with immobilized C1q (Figures 4.1C and 4.1D), soluble BSA or C1q was added to the culture media and incubated for 24 hours. However, no significant morphological changes were observed in cells cultured with soluble C1q as compared to cells cultured with immobilized C1q (Figure 4.2). Given that soluble C1q did not have significant effects on osteoclast differentiation (Figure 3.10), the results obtained here strengthen the link between cellular morphological changes and regulation of monocyte development by immobilized C1q.

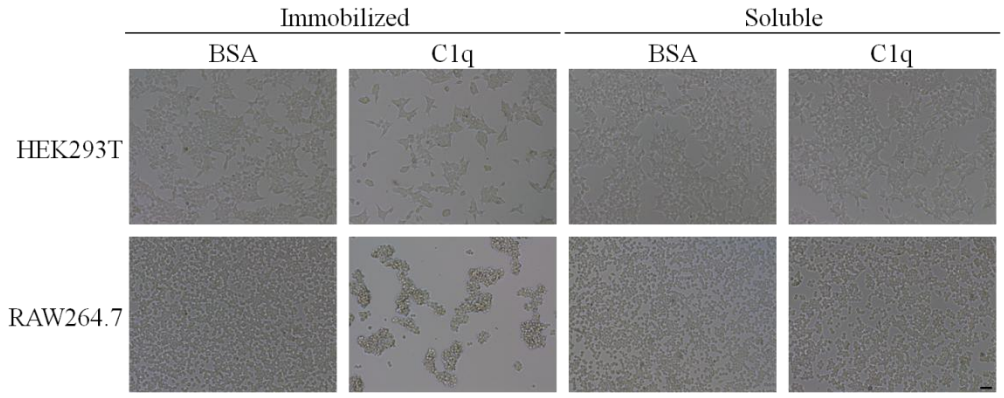


Figure 4.2. Cellular morphologies were not affected by soluble C1q. HEK293T and RAW264.7 cells were cultured in BSA- or C1q-coated wells or in the presence of 50 μg/ml BSA or C1q. Microscopic examinations were then performed on the cells after incubating for 24 hours. Scale bar represents 25 μm. The results are representative of three independent experiments.

4.2.2 Adherence of the cells is associated with the induced morphological changes

As the morphological changes induced by immobilized C1q were found to affect the adherence of DCs (Teh *et al.*, 2011), adherence of the cells was also examined in the presence of immobilized C1q. Cells were incubated in plates coated with BSA or C1q for 24 hours and crystal violet staining was performed to determine the amount of adherent cells.

Monocytes, macrophages and DCs were significantly less adherent in the presence of immobilized C1q (Figure 4.3A). However, the decrease in adherence for imC1q monocytes was lesser than that of imC1q macrophages and DC as compared to their respective controls (Figure 4.3A). This can be correlated to the reduced morphological changes observed in imC1q monocytes as compared to that of imC1q macrophages and DC with their respective control cells (Figure 4.1B).

Similarly, a correlation between morphological changes and adherence by immobilized C1q can be observed in the human and murine cell lines (Figures 4.3B and 4.3C). All human cell lines showed significant decreases in adherence when incubated with immobilized C1q (Figure 4.3B). HEK293T cells, which exhibited distinct morphological changes among the human cell lines (Figure 4.1C), showed the greatest drop in adherence (Figure 4.3B).

The correlation was further validated by the murine cell lines in which all immune cell lines (DC2.4, J774, RAW264.7 and N9) were significantly less adherent when added to C1q-coated wells (Figure 4.3C). These cell lines also displayed extensive morphological changes as compared to the non-immune cell lines (Figure 4.1D). However, the slight decrease in adherence for C2C12 and NIH-3T3 cells was not significant (Figure 4.3C) and this correlates with their morphological changes which were modest as compared to those of the immune cell lines (Figure 4.1D). Thus, the morphological changes induced by immobilized C1q are highly associated with cell adherence.

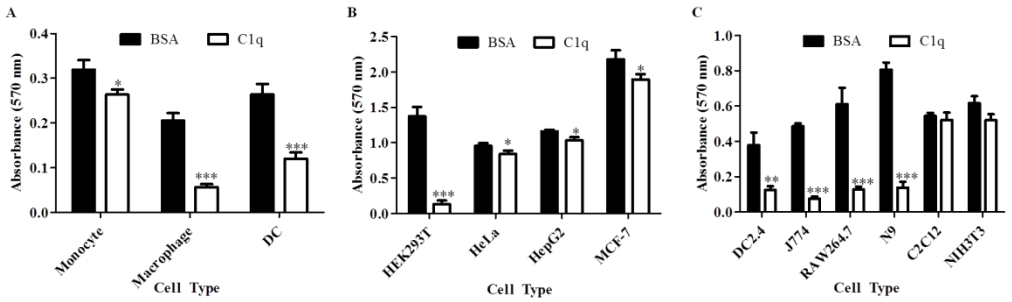


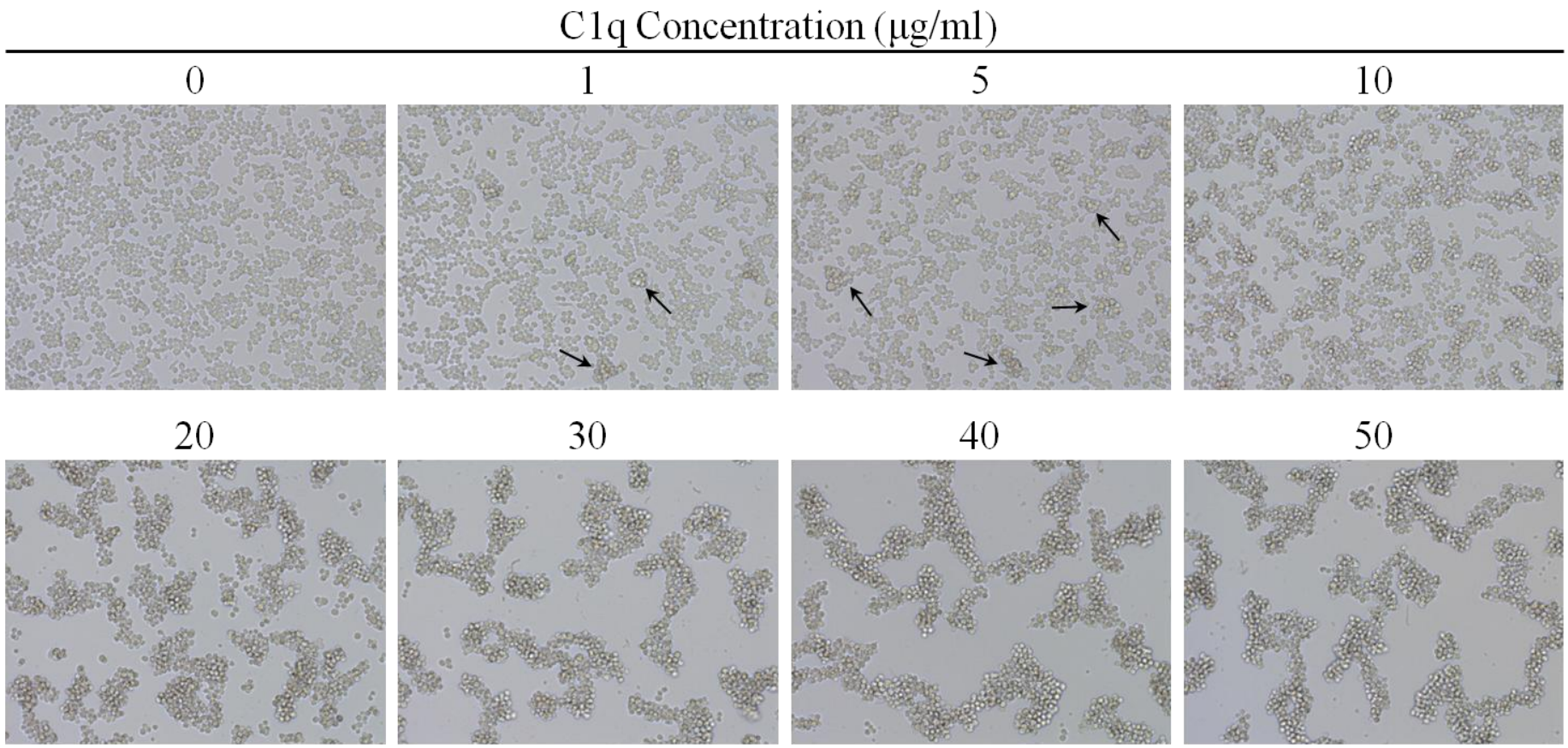
Figure 4.3. Cell adherence is associated with immobilized C1q-induced morphological changes. Cells were incubated in BSA- or C1q-coated wells and incubated for 24 hours. Crystal violet staining was carried out to quantify the number of adherent cells. **(A-C)** Human primary cells, human cell lines and murine cell lines were used for the experiment respectively. Triplicates were performed for each condition and presented as mean ± S.D. The results are representative of three independent experiments.

4.3 Anti-adhesive effects of solid phase C1q is specific

Given that immobilized C1q induced significant morphological changes and affected the adherence of cells, we would like to determine if these effects were specific. Thus, different concentrations of C1q were used to coat the culture plates and the morphologies and adherence of the cells were then observed after 24 hours of culture. Cells cultured with low concentrations of solid phase C1q (1 and 5 μg/ml) exhibited minimal changes in morphologies with some clustering observed (Figure 4.4A). However, with increasing concentrations of solid phase C1q, aggregation of the cells and rounder cells became more obvious (Figure 4.4A). Not surprisingly, the increased morphological changes were accompanied with decreased adherence of these cells (Figure 4.4B). The decrease in adherence was significant when 10 μg/ml C1q was used to coat the wells as compared to control cells in uncoated wells (Figure 4.4B). The cells showed decreasing adherence when 20, 30, 40 and 50 μg/ml C1q were used for coating (Figure 4.4B).

To further validate that the effects were specific, C1q was heat inactivated (HI) at 56^{o}C for 30 minutes before coating was carried out. Cells were then incubated for 24 hours and adherence of the cells was determined. Heat inactivation of C1q increased the adherence of the cells as compared to when fresh C1q was used to coat the plate (Figure 4.4C). On the other hand, heat inactivation did not affect the adherence of cells added to BSA-coated wells (Figure 4.4C). To ensure that HI did not affect the coating of C1q on tissue culture plate, coated C1q was eluted and quantified with silver staining. Equal amounts of C1q were detected for fresh and HI C1q-coated wells (Appendix 3). Hence, the results from these experiments suggest that the effects observed by immobilized C1q were specific.

A



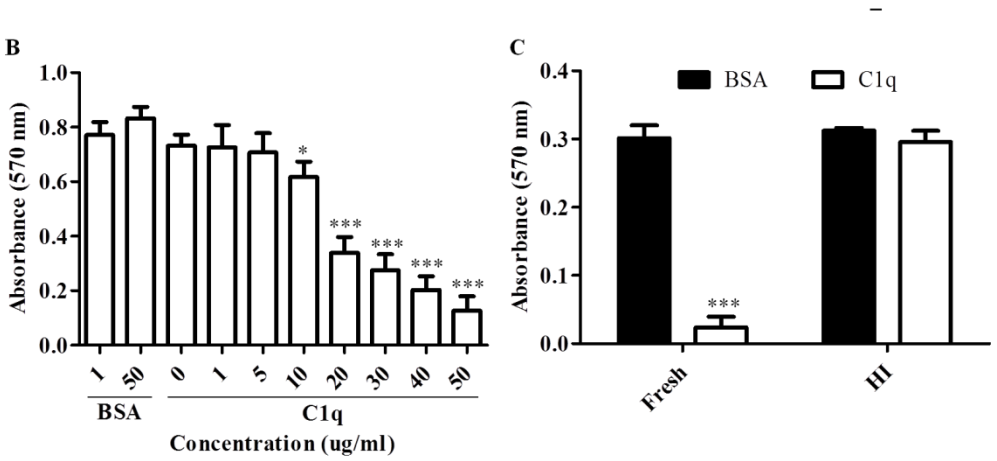


Figure 4.4. Changes in cell morphologies and adherence of the cells mediated by immobilized C1q are specific. (A) Different concentrations of C1q were used to coat the wells before RAW264.7 cells were added. After incubating for 24 hours, microscopic examination was performed to observe the morphologies of the cells. Arrows indicate cell clusters. Scale bar represents 25 μm. **(B)** Similar to A, the amount of adherent cells was determined by crystal violet staining. Statistical analysis was carried out by comparing with C1q-coated wells with uncoated wells. **(C)** BSA and C1q were heat inactivated (HI) before coating was carried out. The cells were then seeded and the amount of adherent cells was quantified after 24 hours with crystal violet staining. For the cell adhesion assay, triplicates were performed for each condition and presented as mean ± S.D. The results are representative of three independent experiments.

4.4 Protein synthesis is not required for the effects mediated by solid phase C1q

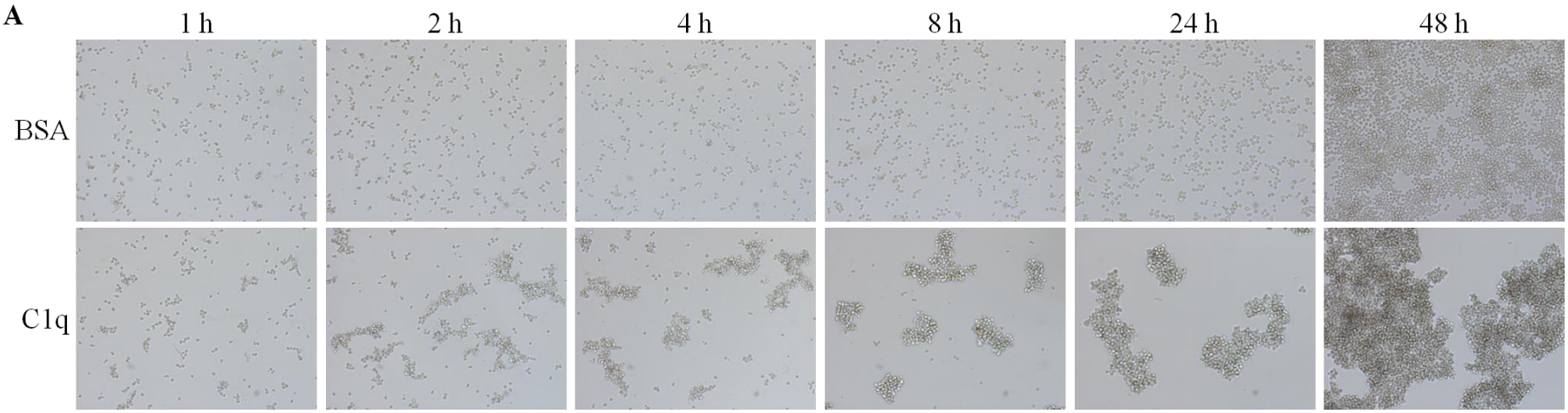
4.4.1 Immediate effects of immobilized C1q on morphologies and adherence of the cells

In order to increase understanding of the mechanisms in which immobilized C1q mediates the effects observed, cells were observed at various time points during culture with immobilized C1q. As seen in Figure 4.5A, aggregation of the cells was observed by 1 hour of incubation with immobilized C1q. The extent of aggregation increased at the later time points and larger cell clusters were also observed (Figure 4.5A). This may be due to either the clustering of various small cell aggregates, the proliferation of cells which increases the sizes of the aggregates or both. The adherence of the cells was also determined and decreased adherence was observed at all the time points (Figure 4.5B). These data further validated previous observations in which morphological changes in the cells were highly associated with their adherence (Figures 4.1 and 4.3).

4.4.2 Inhibition of protein synthesis does not reverse the effects of immobilized C1q

As the effects mediated by immobilized C1q was immediate, this suggests that protein synthesis is not involved in the process. To verify this, CHX, an inhibitor of protein synthesis, was used. Cells were pre-treated with different concentrations of CHX before adding into culture plates coated with BSA or C1q. The inhibition of protein synthesis did not abrogate the decreased adherence of cells by immobilized C1q (Figure 4.5C). Instead, decreased absorbance was observed when cells were pre-treated with 0.1 μg/ml CHX and incubated with immobilized BSA at the later time points. This is mainly due to the toxic effects of CHX as apoptotic cells were observed in these cultures (data not shown). To show that CHX was effective in inhibiting protein synthesis, CHX pre-treated cells were stimulated with IFNγ and western blot

was carried out. Upon stimulation with IFNγ, Stat1 was up-regulated but this up-regulation was inhibited when the cells were pre-treated with CHX (Figure 4.5D). Hence, protein synthesis is not required to mediate the effects of immobilized C1q.


A
1 h
2 h
4 h
8 h
24 h
48 h
BSA
C1q

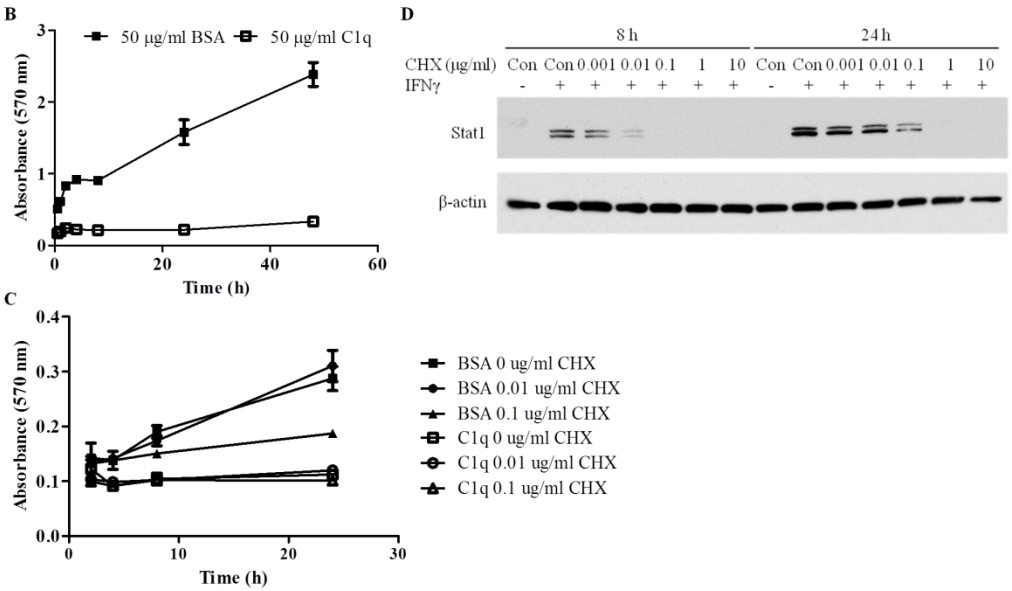


Figure 4.5. Immobilized C1q had an immediate effect on the cells which did not require protein synthesis. (A and B) RAW264.7 cells were seeded into BSA- or C1q- coated wells and incubated for various time points. Microscopic examination **(A)** and crystal violet staining **(B)** were performed at each time point to observe the morphological changes and cell adherence respectively. Scale bar represents 25 µm. **(C)** The cells were pre-treated with cycloheximide (CHX) and added to BSA- or C1q- coated wells. At different time points, crystal violet staining was carried out to determine the amount of adherent cells. **(D)** Cells pretreated with CHX were stimulated with IFNγ and incubated for 8 or 24 hours. Western blotting was done to determine the expression of Stat1. The results are representative of three independent experiments.

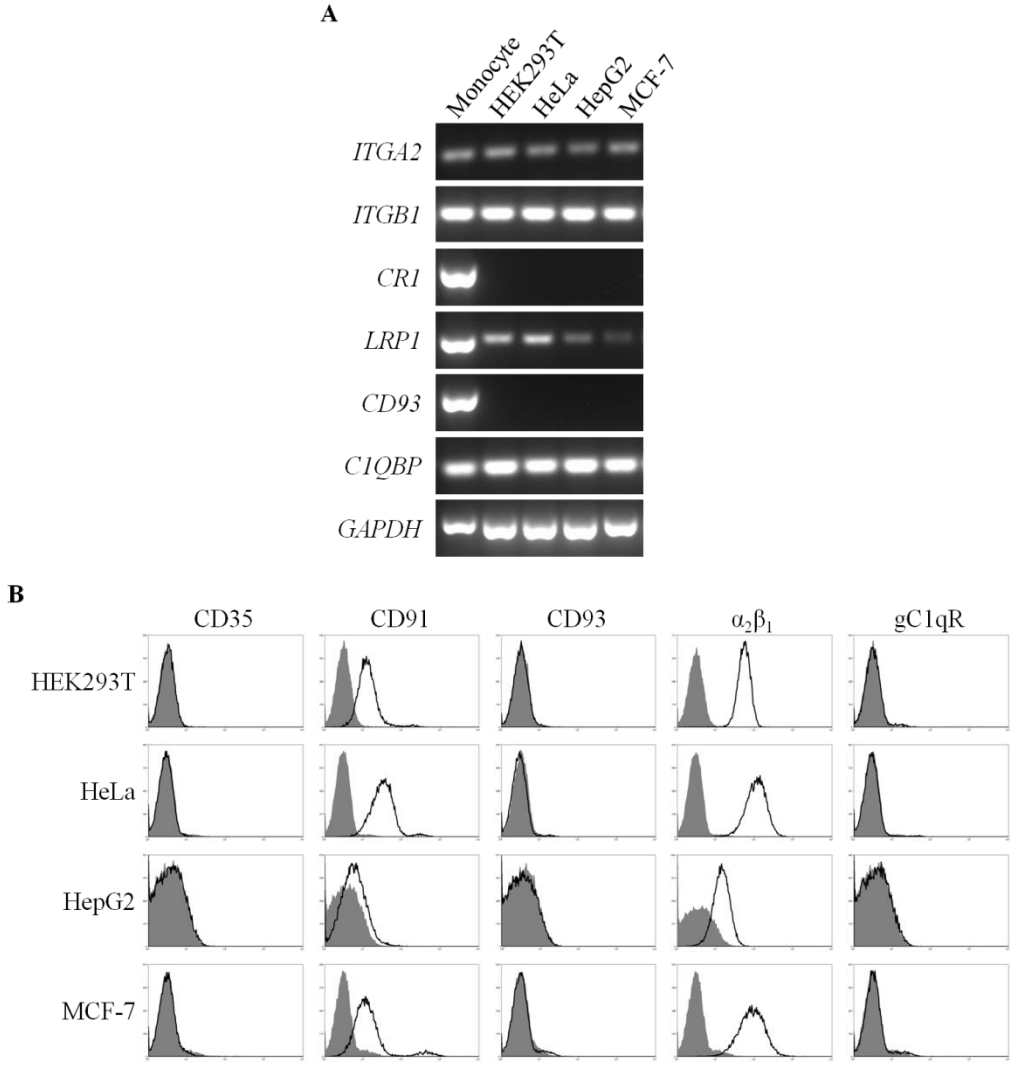
4.5 Known C1q receptors are not likely to be involved in the effects of immobilized C1q

Given that protein synthesis is not required for immobilized C1q to exert its effects, existing C1q receptors should be involved in mediating the effects. Studies have identified many different C1q receptors which are important in mediating various cellular functions (Lu et al., 2008). Previously, we showed that HEK293T cells had the greatest changes in their morphologies and adherence in the presence of immobilized C1q whereas the remaining human cell lines exhibited moderate changes in their morphologies and adherence (Figures 4.1 and 4.2). Similar observations were also made for the murine cell lines. As these cell lines displayed varying morphological changes and adherence with immobilized C1q, it is interesting to investigate if there is any correlation between the expression of these known C1q receptors and the phenotypes observed. Using the different human cell lines, RT-PCR and flow cytometry were used to determine the expression of these receptors at the mRNA and protein levels respectively. At the mRNA level, $\alpha_2\beta_1$ (*ITGA2* and *ITGB1*), CD91 (*LRP1*) and gC1qR (*C1QBP*) were detected in all cell lines (Figure 4.6A). However, no distinct correlation between the expression of these receptors and the observed phenotypes was found.

Next, surface staining using various antibodies specific against the C1q receptors was performed. $\alpha_2\beta_1$ and CD91were detected on the cell surfaces (Figure 4.6B). gC1qR was not detected on the surfaces of these cells (Figure 4.6B) but the presence of gC1qR was detected via intracellular staining (data not shown). This is not surprising as gC1qR is known to be an intracellular molecule (Dedio *et al.*, 1998; van Leeuwen and O'Hare, 2001). Similarly, no correlation was observed between the surface expression of C1q receptors and immobilized C1q-induced phenotypes.

To determine if the receptors present on the human cell lines were involved in the effects observed, antibodies specific for these receptors were used to block the receptors on the cells before incubating in C1q-coated wells. HEK293T cells were used in this experiment as they do not express Fc

receptors and this will not affect interpretation of the results. As shown in Figure 4.6C, blocking of these receptors did not abrogate the effects mediated by immobilized C1q and the cells remained clustered and rounded. Thus, $\alpha_2\beta_1$, CD35, CD91, CD93 and gC1qR are not likely to be responsible for the effects of immobilized C1q.



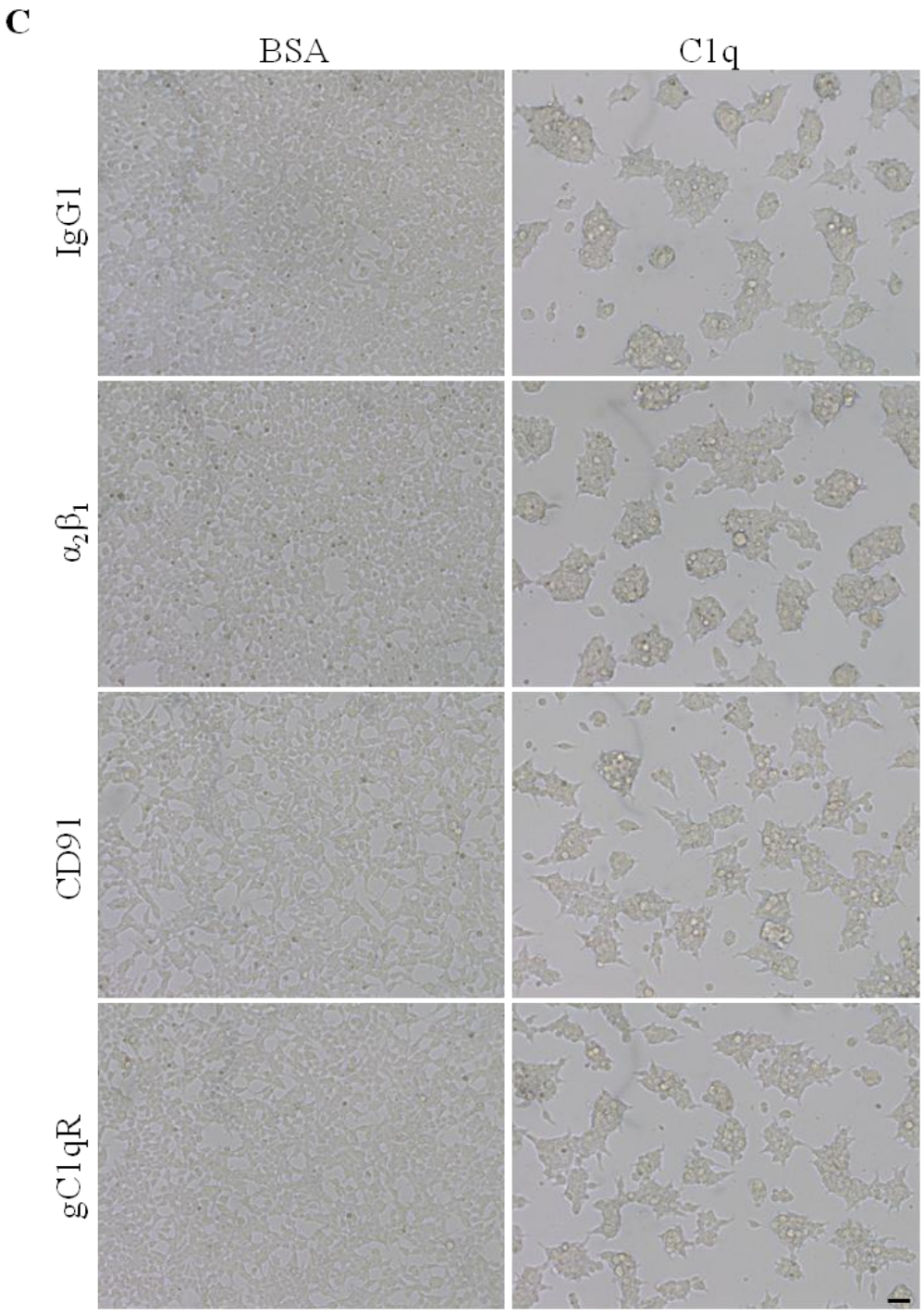


Figure 4.6. Known C1q receptors are not involved in mediating the effects of immobilized C1q. (A) The expression of C1q receptors were examined at the mRNA level. RT-PCR was performed to determine the levels of C1q receptors for each cell line. Human primary monocytes were used as a positive control. *GAPDH* was used as an endogenous control. **(B)** Surface expression of C1q receptors were determined by surface staining and analyzed by flow cytometry. **(C)** Antibodies specific against $\alpha_2\beta_1$, CD91 and gC1qR which are present on HEK293T cells were used to block the receptors. The cells were then added into BSA- or C1q-coated wells and examined under the microscope after 24 hours. Scale bar represents 25 μm. The results are representative of three independent experiments.

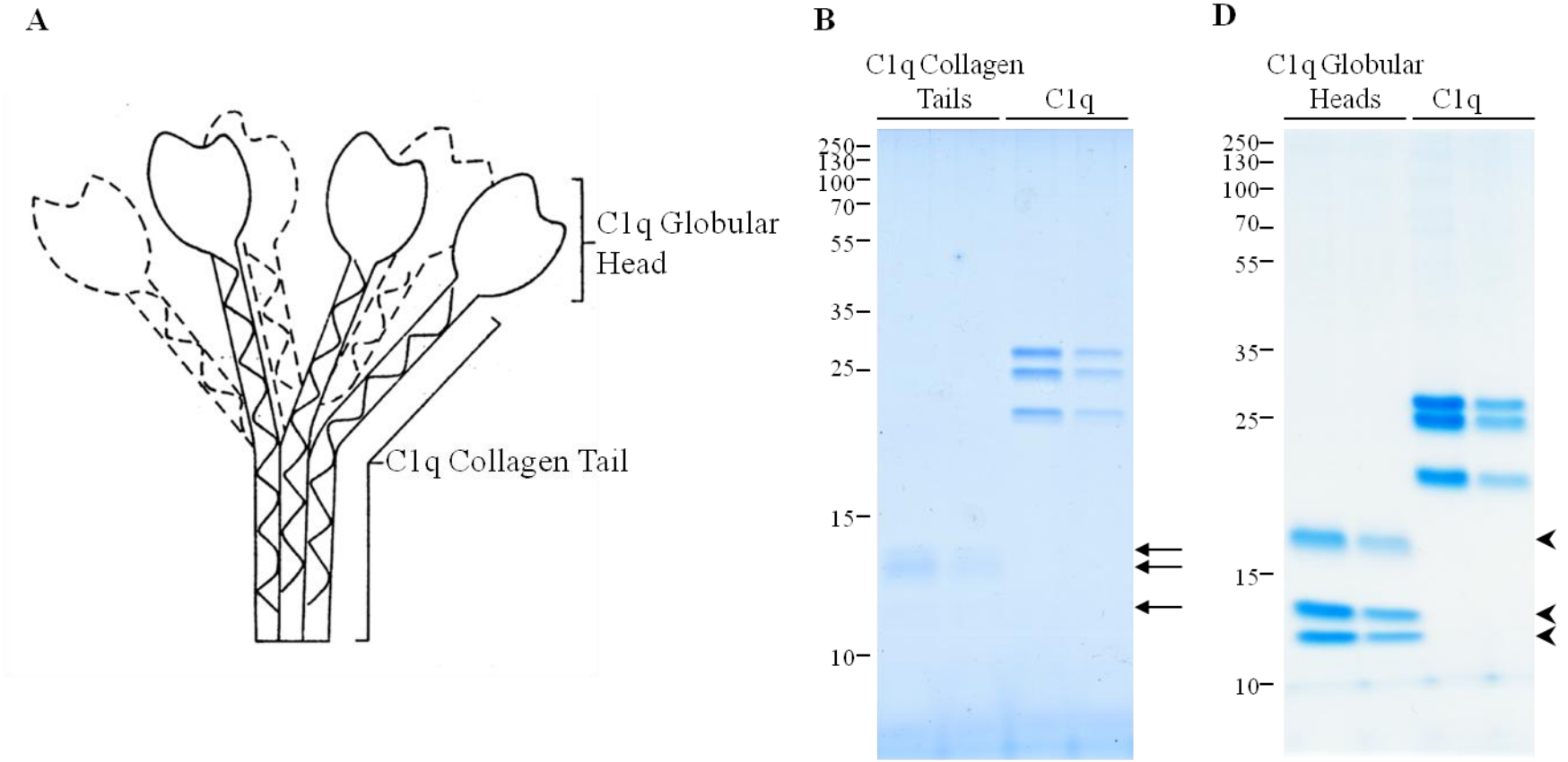
4.6 Augmentation of the morphological changes by anti-C1q antibodies

4.6.1 Intact C1q is required to induce changes in the morphologies of cells

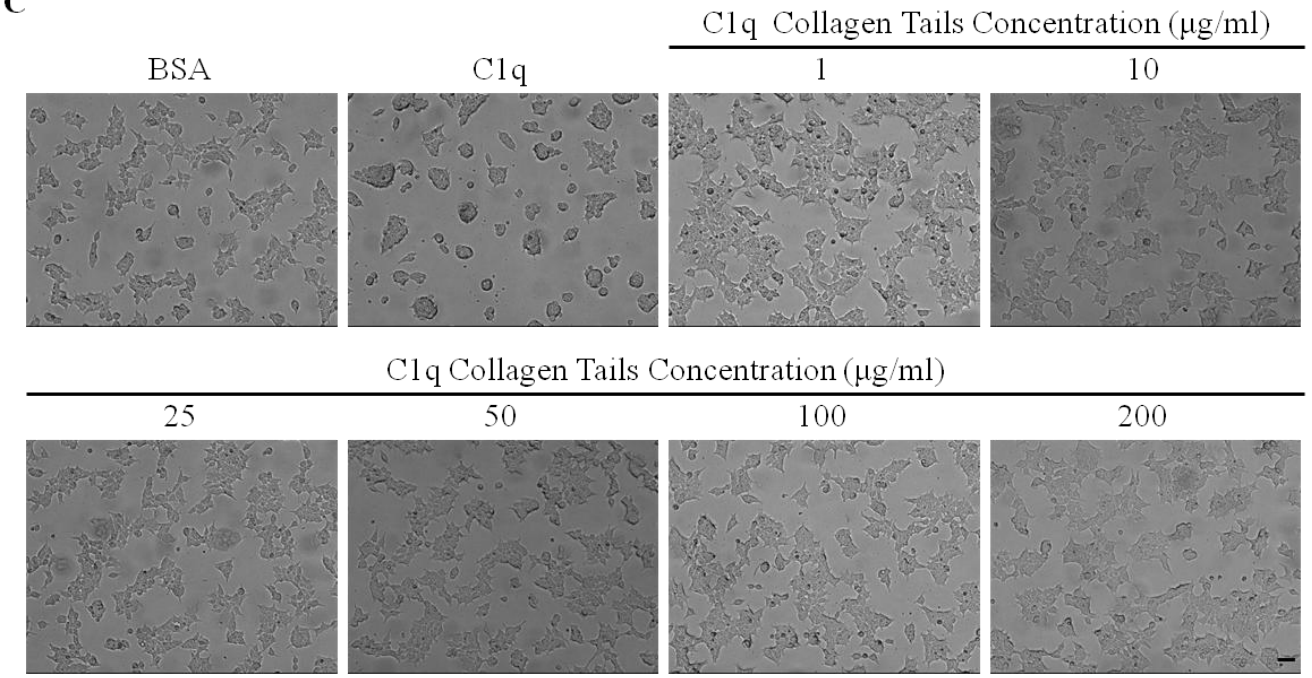
The functional C1q molecule is made up of 18 polypeptide chains (6 A, 6 B and 6 C chains) and consisted of globular heads and collagen tails (Figure 4.7A) (Lu *et al.*, 2008; Reid and Porter, 1976). It is of interest to understand if the effects observed for immobilized C1q were mediated by the different domains of C1q. This may lead to the identification of receptors that interact with either the globular heads or collagen tails of C1q.

Collagen tails were generated by pepsin digestion of C1q which removed the globular heads. FPLC was carried out to purify the collagen tails and the purity was determined by Coomassie Blue staining (Figure 4.7B). After obtaining pure C1q collagen tails, various concentrations were used to coat culture plates and cells were cultured for 24 hours. No obvious morphological changes were observed on cells cultured with C1q collagen tails (Figure 4.7C). This is in contrast to the cell clusters and rounded cells that were observed in cells cultured with immobilized C1q (Figure 4.7C).

Next, the role of C1q globular heads in mediating the effects of immobilized C1q was determined. Globular heads were generated by the collagenase digestion of C1q which removed the collagen tails. FPLC was carried out to purify the globular heads and the purity was determined by Coomassie Blue staining (Figure 4.7D). After obtaining pure C1q globular heads, various concentrations were used to coat culture plates and cells were cultured for 24 hours. Similarly, the globular heads did not affect the morphologies of the cells whereby the cells remained flat and extended (Figure 4.7E). Thus, the results indicate that intact C1q is required to mediate the effects of immobilized C1q.


A
C1q Globular Head
C1q Collagen Tail
B
C1q Collagen Tails
C1q
250
130
100
70
55
35
25
15
10
D
C1q Globular Heads
C1q
250
130
100
70
55
35
25
15
10

C


C1q Collagen Tails Concentration (µg/ml)
BSA
C1q
1
10
C1q Collagen Tails Concentration (µg/ml)
25
50
100
200

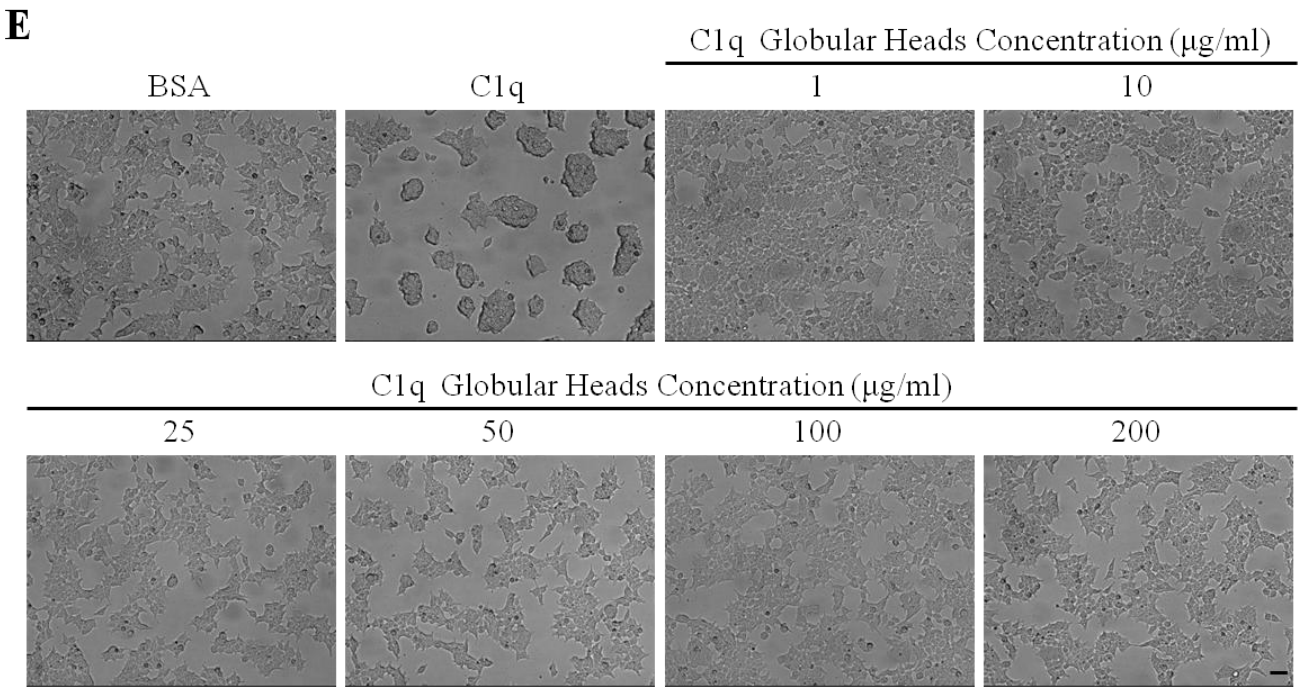


Figure 4.7. Intact C1q is essential for the morphological changes mediated by immobilized C1q. (A) Schematic diagram of the globular heads and collagen tails of C1q. The figure is adapted from Reid and Porter, 1976. **(B)** Collagen tails were obtained by pepsin digestion of C1q followed by FPLC purification. The purity of the collagen tails was determined by Coomassie Blue staining. Arrows indicate the collagen domains of three C1q chains. **(C)** Different concentrations of collagen tails were used to coat the wells before HEK293T cells were added. After incubating for 24 hours, microscopic examination was performed to observe the morphologies of the cells. **(D)** Globular heads were obtained by collagenase digestion of C1q followed by FPLC purification. The purity of the globular heads was determined by Coomassie Blue staining. Arrowheads indicate the globular domains of three C1q chains. **(E)** Similar to **C** except that globular heads were used to coat the wells instead. Scale bar represents 25 μm. The results are representative of three independent experiments.

4.6.2 Anti-C1q antibodies enhanced the morphological changes by immobilized C1q

Previously, we have shown that immobilized C1q, but not soluble C1q, is required to mediate the changes in morphology and adherence of the cells (Figures 4.2 and 4.3). Thus, this could be due to effective cross-linking of C1q receptor(s) by immobilized C1q as compared to soluble C1q or exposure of conformation-dependent binding sites on immobilized C1q. However, given that intact C1q but not high amounts of globular heads and collagen tails is required to mediate these changes (Figure 4.7), it is likely that neoepitopes exposed during C1q immobilization are involved in these changes. Studies have shown that some SLE patients have anti-C1q antibodies and these antibodies only recognize immobilized but not soluble C1q (Antes *et al.*, 1988; Uwatoko *et al.*, 1987; Wener *et al.*, 1989). Hence, we would like to determine if the blocking of such epitopes by anti-C1q antibodies is able to abrogate the effects observed for immobilized C1q.

Commercial anti-C1q antibodies were first used to test the hypothesis. Polyclonal goat anti-C1q antibody was used to block the immobilized C1q before HEK293T cells were seeded into the wells. Goat IgG and a non-specific antibody, goat anti-C1s, were used as controls. After 1 hour, the cells in C1q-coated exhibited rounded morphology whereas those in BSA-coated wells started to have some cellular extensions (Figure 4.8A). The cells remained rounded and cell clusters were obvious at 4-hour time point in the presence of immobilized C1q (Figure 4.8A). The control cells continued to spread and appeared flat after longer incubation (Figure 4.8A). Although the kinetics for HEK293T cells seemed slower as compared to RAW264.7 cells in forming cell clusters (Figure 4.5A), differences between HEK293T cells seeded in BSA- and C1q-coated wells could be observed in the earlier time points (Figure 4.8A). Surprisingly, upon the addition of goat anti-C1q to C1q-coated wells, cells were observed to clump at a much earlier time point (Figure 4.8A). Goat anti-C1q also induced the formation of larger but lesser cell clusters in C1q-coated wells after 24 hour of incubation (Figure 4.8A). In contrast, isotype and

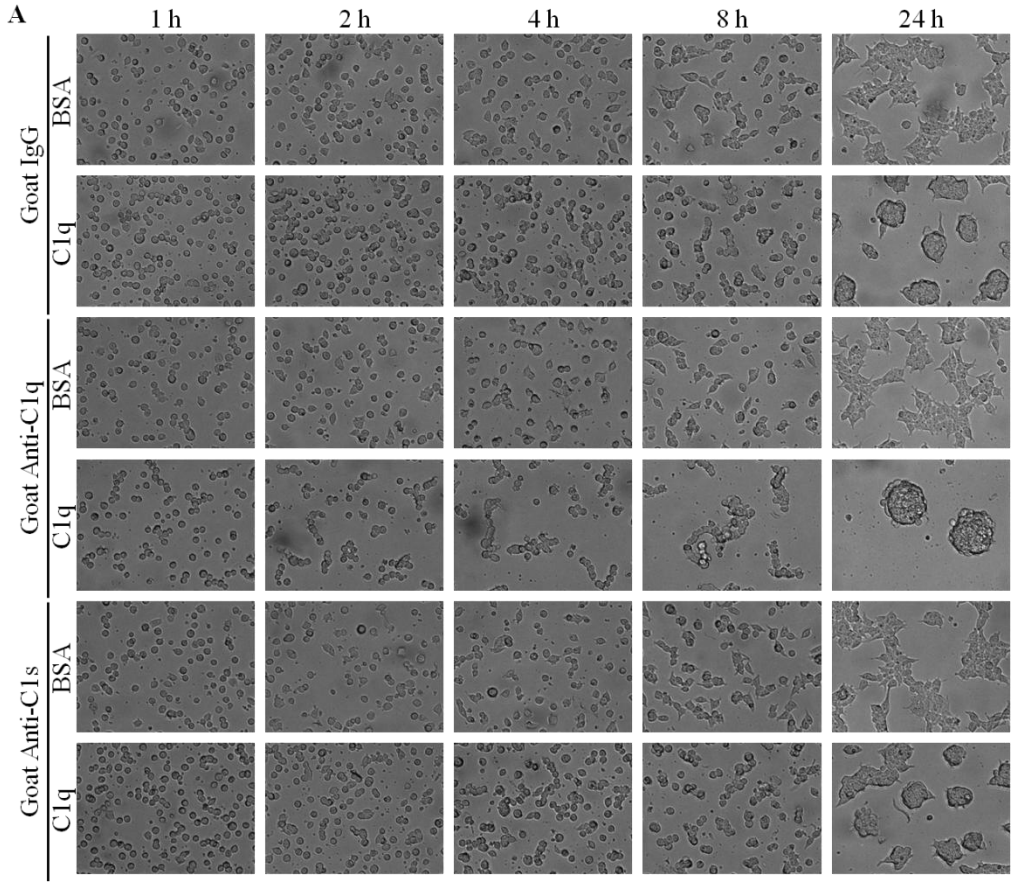
non-specific antibodies did not enhance the morphological changes of cells in both BSA- and C1q-coated wells at different time points (Figure 4.8A).

Next, a monoclonal mouse anti-C1q antibody which is specific against the globular heads of C1q was used. Similar to the polyclonal goat anti-C1q antibody, binding of antibody to immobilized C1q induced faster formation of cell clusters as compared to the isotype control (Figure 4.8B). Larger cell clusters were also observed at the later time points (Figure 4.8B). Cells incubated in BSA-coated wells remained flat and well spread out no matter whether isotype or mouse anti-C1q antibody was added (Figure 4.8B). To ensure specific binding of the antibodies to immobilized C1q, ELISA was performed and both polyclonal and monoclonal anti-C1q antibodies displayed binding to C1q-coated wells but not BSA-coated wells (Figure 4.8C). The other control antibodies exhibited little binding to both BSA- and C1q-coated wells, indicating that the anti-C1q antibodies are specific (Figure 4.8C).

Given that the commercial antibodies enhanced the effects of immobilized C1q, the effects of anti-C1q autoantibodies in SLE patients were also investigated. Plasma of SLE patients were screened using ELISA in which high salt was used to prevent the binding of immune complexes to the C1q-coated wells. As seen in Figure 4.9A, all patients showed higher readings for C1q-coated wells as compared to control wells which were not coated. Furthermore, the amount of anti-C1q autoantibodies was higher in all samples as compared to the negative control (Figure 4.9A). 10 samples with the highest readings were then chosen, pooled and anti-C1q autoantibodies were concentrated by IgG precipitation. After concentrating the samples, the IgG fraction, which contained anti-C1q autoantibodies, showed higher reading as compared to most of the original samples except Patient D which still had a higher reading (Figure 4.9B).

The human anti-C1q antibody was used to block immobilized C1q and the effects on the cells were determined. In contrast to the commercial antibodies, human anti-C1q did not augment the morphological changes of cells incubated in C1q-coated wells (Figure 4.9C). No distinct differences were

observed between the isotype and human anti-C1q antibody for cells added to BSA- or C1q-coated wells (Figure 4.9C). To ensure that there is binding between the human anti-C1q autoantibodies and immobilized C1q, ELISA was carried out and the results indicated binding of the autoantibodies to C1q-coated wells (Figure 4.9D). Thus, the autoantibodies of SLE patients do not inhibit or enhance the effects of immobilized C1q.



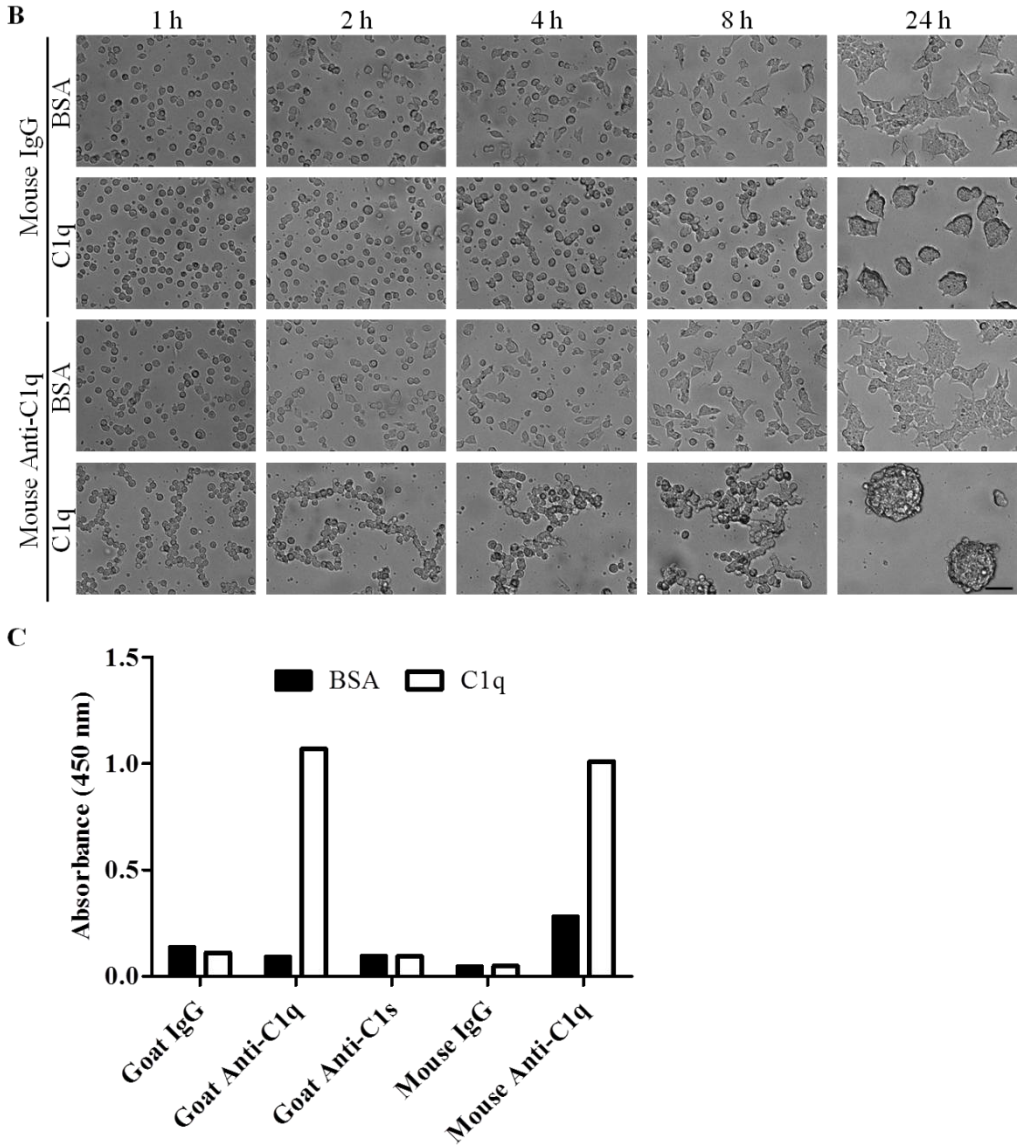


Figure 4.8. Anti-C1q antibodies enhanced the morphological changes mediated by immobilized C1q. (A and B) Tissue culture plates were first coated with BSA or C1q. The plates were then blocked and subsequently incubated with various antibodies including goat anti-C1q (**A**) and mouse anti-C1q (**B**). Goat IgG, goat anti-C1s and mouse IgG were used as controls. Cells were then added to the wells and microscopic images were taken at various time points. Scale bar represents 25 μm. **(C)** ELISA was performed to ensure that the antibodies were binding specifically to immobilized C1q. High salt (1M NaCl) was used in the ELISA to prevent the binding of immune complexes to immobilized C1q. The results are representative of three independent experiments.

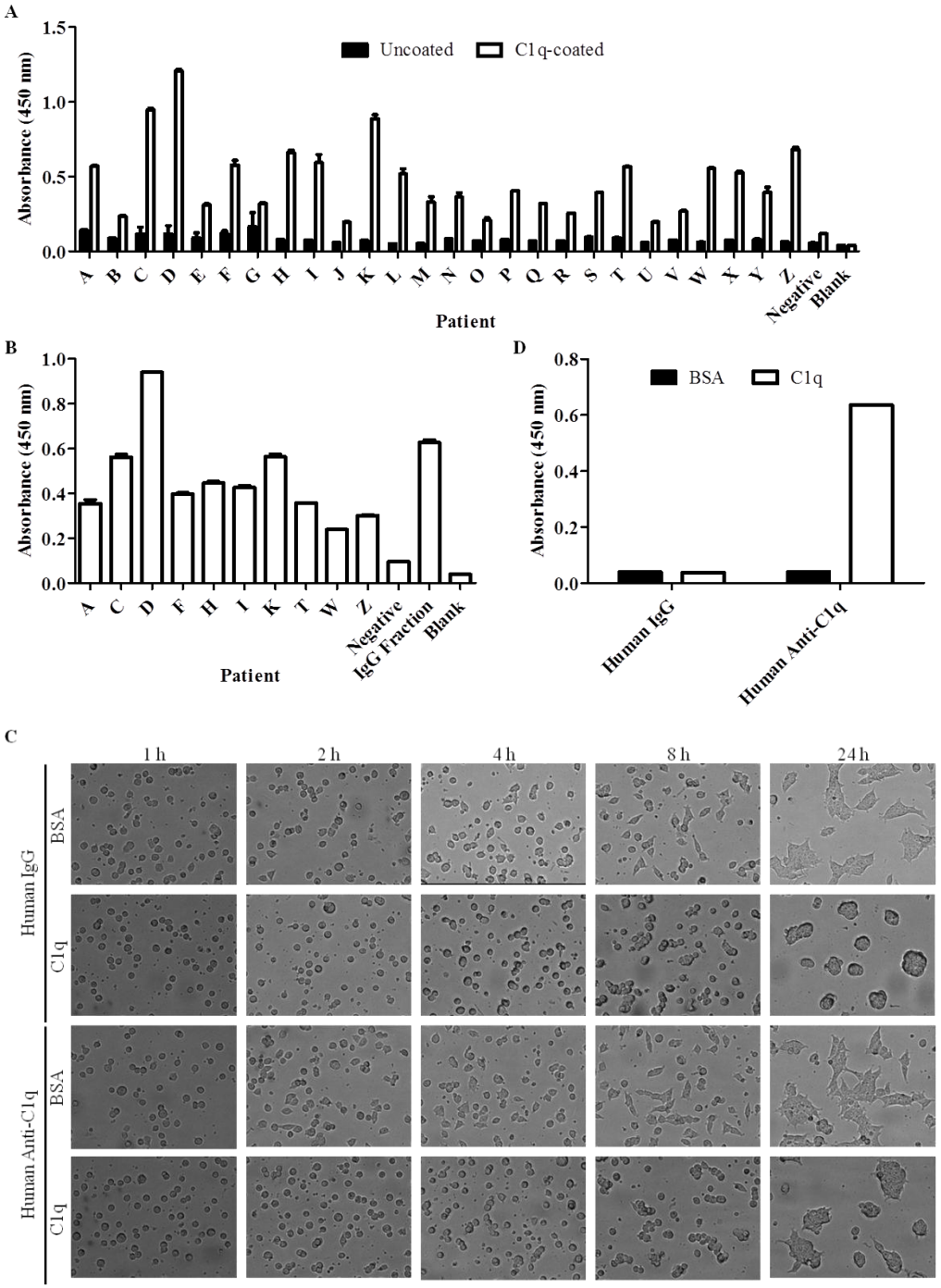


Figure 4.9. Anti-C1q antibodies of SLE patients do not enhance the morphological changes of immobilized C1q. (A) SLE patients were screened for the presence of anti-C1q antibodies in their plasma. High salt (1M NaCl) was used in the ELISA to prevent the binding of immune complexes to immobilized C1q. To ensure the specificity of the assay, wells without C1q-coating were also used. **(B)** Plasma of 10 patients with high concentrations of anti-C1q antibodies were pooled and concentrated via IgG precipitation. The IgG fraction was then compared with the original samples to ensure the samples were concentrated. **(C)** Tissue culture plates were first coated with BSA or C1q. The plates were then blocked and incubated with human IgG or human anti-C1q (IgG fraction) before the adding of cells to the wells. Microscopic examination was performed at indicated time points. Scale bar

represents 25 μm. **(D)** ELISA was performed to ensure specific binding of antibodies to immobilized C1q. The results are representative of three independent experiments.

Chapter 5 – Discussion

5.1 Production of C1q by osteoclasts

Macrophages exist in different organs and they exhibit diverse phenotypes and functions (Gordon and Taylor, 2005). Derived from monocytes, they are phagocytes which help in the clearance of pathogens and apoptotic cells (Dempsey *et al.*, 2003; Lu *et al.*, 2008). Although multiple studies have shown C1q biosynthesis by some subsets of macrophages (Armbrust *et al.*, 1997; Haga *et al.*, 1996; Loos *et al.*, 1980; Loos *et al.*, 1981; Schafer *et al.*, 2000; Tripodo *et al.*, 2007), it is still unknown if the production of C1q is a common characteristic of macrophages. Local synthesis of C1q is critical as the availability of serum C1q to tissues remains unknown. This can be seen in arteries, brain, esophagus, liver and placenta in which C1q is produced locally (Agostinis *et al.*, 2010; Armbrust *et al.*, 1997; Bobryshev *et al.*, 2010; Bulla *et al.*, 2008; Cao *et al.*, 2003; Schafer *et al.*, 2000; Stevens *et al.*, 2007). As C1q regulates many functions and is involved in both health and diseases (Lu *et al.*, 2008; Nayak *et al.*, 2010), it is important to know its production so as to further understand how it modulates these diverse functions.

In this study, the production of C1q by osteoclasts and the potential role of C1q in the bone microenvironment are investigated. *In vitro* osteoclasts were first differentiated from monocytes using M-CSF and RANKL and subsequently characterized. These osteoclasts exhibited characteristic osteoclast features which include expression of osteoclast markers such as CTR, CAII, CatK, MMP-9 and TRAP, presence of F-actin rings and most importantly, the ability to resorb bone (Figure 3.2). In addition, these cultured osteoclasts possessed immune molecules which are involved in antigen presentation (Figure 3.3) and this explains their role in activating the adaptive immunity (Grassi *et al.*, 2011; Kiesel *et al.*, 2009; Li *et al.*, 2010). The production of C1q by *in vitro* osteoclasts was clearly demonstrated at both the transcriptional and translational levels (Figure 3.4 and 3.5). These results were further validated through immunohistochemistry of human femoral bone samples by showing the expression of C1q in endogenous osteoclasts (Figures

3.8 and 3.9). Thus, osteoclasts produce C1q, adding osteoclasts to the list of macrophages that produce C1q (Armbrust *et al.*, 1997; Haga *et al.*, 1996; Loos *et al.*, 1980; Loos *et al.*, 1981; Schafer *et al.*, 2000; Tripodo *et al.*, 2007) and this increases the possibility of C1q being a common trait for different subsets of macrophages. Further studies involving extensive characterization of other subsets such as red-pulp macrophages, white-pulp macrophages and metallophilic macrophages or the generation of a transgenic C1q mouse are required to validate this hypothesis.

Mononucleated and multinucleated osteoclasts, representing preosteoclasts and mature osteoclasts respectively, were observed both *in vitro* and *in vivo* (Figures 3.2, 3.6, 3.8 and 3.9). C1q was mainly detected in cultured preosteoclasts by immunofluorescence staining (Figures 3.6 and 3.7). However, upon IFNγ stimulation, C1q was also expressed in mature osteoclasts though the expression level was still lower than that of preosteoclasts (Figure 3.7). The lack of co-localization with endosomal marker EEA-1 suggests that C1q is synthesized *de novo* by both types of osteoclasts (Figures 3.6 and 3.7). In contrast, expression of C1q was detected in both endogenous preosteoclasts and mature osteoclasts (Figures 3.8 and 3.9). This may reflect the imperfect culture conditions for the *in vitro* osteoclasts and such observations were also made in an earlier study involving chondrocytes (Bradley *et al.*, 1996).

5.2 Regulation of C1q production in osteoclasts

The production of C1q can be regulated by multiple factors such as cytokines, drugs and TLR ligands (Lu *et al.*, 2008). It also depends largely on the origin of the cells used in the study. This is reflected by IFNγ where conflicting data exists and depends on the cells used. For example, IFNγ reduced C1q synthesis and secretion in rat Kupffer cells (Armbrust *et al.*, 1997). Furthermore, C1q mRNA level and secretion were inhibited in non-stimulated and thioglycollate-activated murine peritoneal macrophages by IFNγ (Faust and Loos, 2002). However, IFNγ induced a dose-dependent increase in C1q mRNA level and secretion in paraffin oil-activated murine peritoneal macrophages (Zhou *et al.*, 1991a). In addition, IFNγ increased both C1q mRNA level and secretion in murine microglial cells (Haga *et al.*, 1996). A similar increase in C1q production was also observed in human THP-1 derived macrophages (Walker, 1998), human monocyte-derived macrophages (Chen *et al.*, 2011; Kaul and Loos, 2001) and human monocyte-derived DCs (Chen *et al.*, 2011).

Our study clearly demonstrated that IFNγ increased the levels of C1q at the transcriptional and translation levels for primary osteoclasts (Figures 3.7A, 3.7B and 3.7C). These were accompanied with increased C1q secretion by osteoclasts (Figure 3.7D). Similar results were also obtained primary macrophages and DCs (Figure 3.7). These results generally correspond to the data observed in IFNγ-stimulated human cells (Chen *et al.*, 2011; Kaul and Loos, 2001; Walker, 1998). The physiological relevance of C1q induction in osteoclasts by IFNγ remains unknown but could potentially play a role during local bone infection or inflammation which will be discussed later.

TLR ligands also exhibit varying effects on C1q production in different cell types (Lu *et al.*, 2008). LPS, lipoteichoic acid (LTA) and peptidoglycan (PGN) enhanced C1q secretion in human monocyte-derived DCs though LPS had a greater effect than that of LTA and PGN (Baruah *et al.*, 2006). In addition paraffin oil-activated murine peritoneal macrophages exhibited increased C1q mRNA and secretion after stimulation with LPS and C3b-

opsonized zymosan (Zhou *et al.*, 1991b). LPS also increased C1q production in human THP-1 derived macrophages (Walker, 1998) and human monocyte-derived macrophages (Castellano et al., 2004). However, in the same study which showed increased C1q production in human macrophages (Castellano *et al.*, 2004), LPS induced maturation of both human monocyte-derived DCs and $CD34^+$ hematopoietic stem cell-derived DCs and inhibited C1q secretion (Castellano *et al.*, 2004). Similiarly, LPS reduced C1q synthesis and secretion in rat Kupffer cells (Armbrust *et al.*, 1997). No difference in C1q mRNA was observed in microglial cells after LPS stimulation as compared to control cells (Haga *et al.*, 1996).

Given the diverse effects of LPS on C1q production, it is not surprising to observe varying effects of LPS in our study. In general, the expressions of *C1QA, C1QB* and *C1QC* were upregulated by LPS in osteoclasts, macrophages and DCs (Figure 3.7A). However, the upregulation in mRNA was only accompanied by a slight increase in C1q protein synthesis (Figures 3.7B and 3.7C). C1q secretion was not affected by LPS stimulation in macrophages and DCs though a significant difference was observed in osteoclasts (Figure 3.7D). Existing data (Lu *et al.*, 2008) and our results indicate that although C1q is produced by different cell types, regulation of its production might be different in spite of the same stimulant used. This further reflects the diversity of cell types and the effect of microenvironment might have on the local synthesis of C1q.

5.3 Regulation of osteoclastogenesis by C1q

Osteoclastogenesis is a process in which cells of the monocyte/macrophage lineage differentiate into osteoclasts near the bone surface (Figure 5.1) (Boyle *et al.*, 2003; Teitelbaum and Ross, 2003). The process is highly dependent on two cytokines, M-CSF and RANKL. Cells of the monocyte/macrophage lineage are first recruited from the blood. M-CSF and RANKL, produced by mesenchymal cells or osteoblasts, induce the activation of precursor cells through their respective receptors M-CSFR and RANK to differentiate into mononucleated osteoclasts or preosteoclasts. This also induces the activation of transcription factor NFATc1, the master regulator of osteoclastogenesis, leading to the expression of osteoclast markers such as CatK, CTR and TRAP. Multinucleated osteoclasts or mature osteoclasts are subsequently formed by the fusion of preosteoclasts. For bone resorption to occur, further activation of the mature osteoclasts by RANKL is required which leads to the initiation of bone remodeling.

We have shown the preferential expression of C1q in preosteoclasts (Figures 3.6 and 3.7C) which suggests the involvement of C1q in osteoclastogenesis. Hence, the effects of C1q on osteoclastogenesis were investigated. Using both immobilized and soluble C1q, we found that immobilized C1q enhanced the expression of various osteoclast markers (Figure 3.10). In contrast, soluble C1q did not have any significant effect in influencing osteoclast differentiation (Figure 3.10). Upon further examination, immobilized C1q was also shown to increase the expression of *CSF1R* and *TNFRS11A* (Figure 3.10). These two genes encode for M-CSFR and RANK respectively and could potentially explain the increased expression of osteoclast markers in imC1q osteoclasts by making the cells sensitive to the cytokines used for differentiation. In addition, the expression of *IL6* was upregulated in imC1q osteoclasts (Figure 3.10). IL-6 is known to promote osteoclastogenesis and this could be another reason for enhanced osteoclastogenesis observed for immobilized C1q.

Immobilized C1q also affected the expression of several genes which may negatively affect the differentiation of osteoclasts. *IL10* was significantly upregulated in imC1q osteoclasts (Figure 3.10). Furthermore, the expression of *CSF1* was decreased by immobilized C1q though the downregulation did not reach significance (Figure 3.10). IL-10 is known to inhibit osteoclast differentiation (Evans and Fox, 2007; Mohamed *et al.*, 2007). However, given that osteoclastogenesis is augmented instead of inhibited, this indicates that inhibition of osteoclastogenesis due to the changes in gene expression for IL-10 and M-CSF is overwhelmed by the effects of other positive mediators involved in osteoclastogenesis.

Thus, with the results obtained, we proposed a model for the role of C1q in osteoclastogenesis (Figure 5.1). Precursor cells recruited from the blood are stimulated with M-CSF and RANKL. During the process of differentiating into preosteoclasts, these cells also acquire the ability to produce and secrete C1q. Some of the secreted C1q interacts with the bone surface and gets immobilized. The immobilized C1q binds to an unknown C1q receptor present on the preosteoclasts and activate specific signaling pathway. It is previously reported that the receptors M-CSFR and RANK are sequentially expressed in precursor cells during osteoclast differentiation (Arai *et al.*, 1999). Hence, it is likely that immobilized C1q first increased the expression of M-CSFR and upon binding to M-CSF, RANK expression was subsequently upregulated. Further experiments examining the effects of immobilized C1q on osteoclasts at earlier time points are required to validate the hypothesis. The increased expression of M-CSFR and RANK makes the cells more responsive to M-CSF and RANKL, induces increased NFATc1 activation and enhanced the expression of osteoclast markers such as CTR, CAII, CatK and TRAP. Mature osteoclasts decrease their C1q production and this may represent an intrinsic regulation of osteoclastogenesis, preventing the formation of excessive osteoclasts.

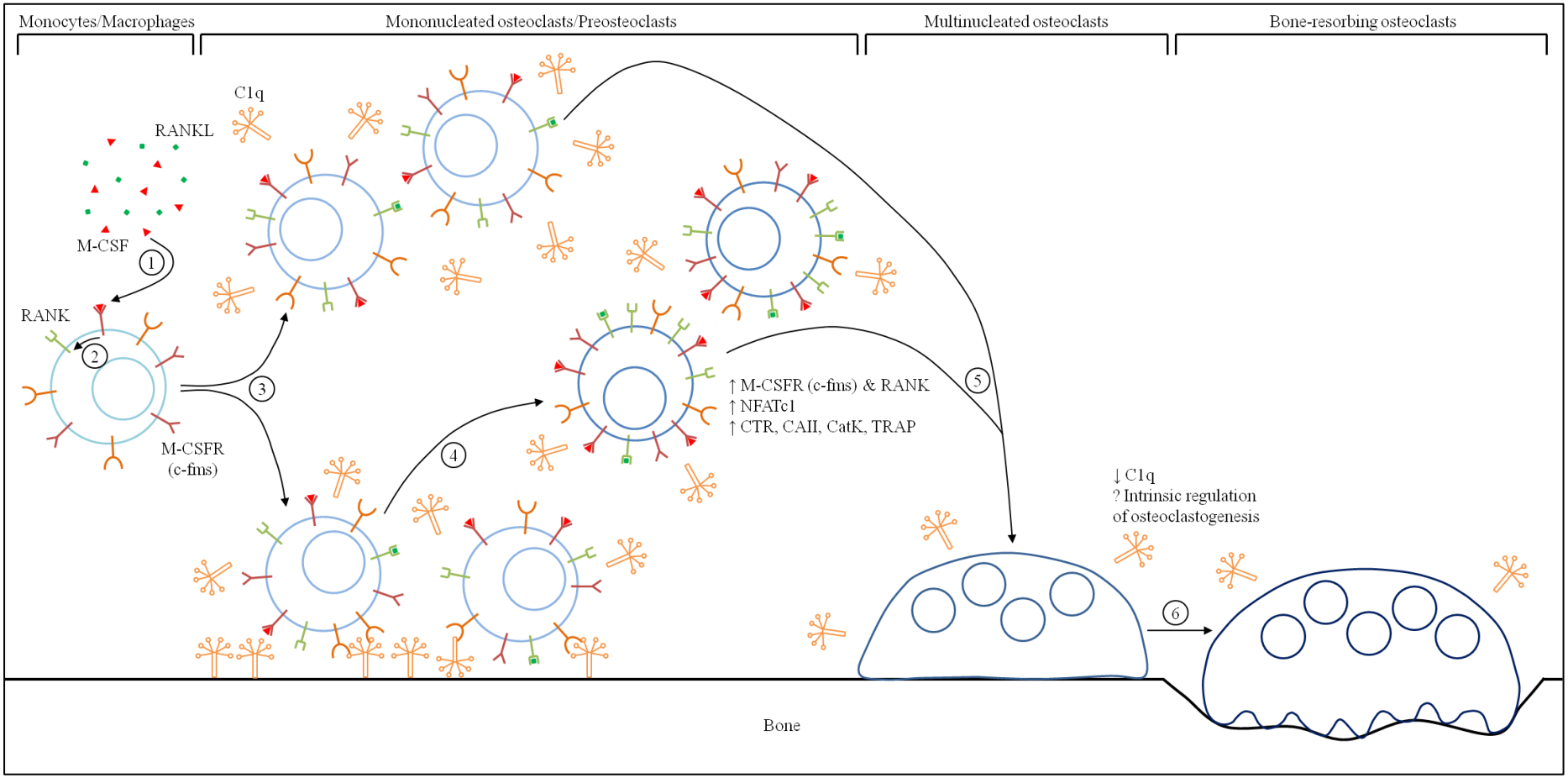

Monocytes/Macrophages
Mononucleated osteoclasts/Preosteoclasts
Multinucleated osteoclasts
Bone-resorbing osteoclasts
C1q
RANKL
M-CSF
1
RANK
2
M-CSFR
(c-fms)
3
4
↑ M-CSFR (c-fms) & RANK
↑ NFATc1
↑ CTR, CAII, CatK, TRAP
5
↓ C1q
? Intrinsic regulation
of osteoclastogenesis
6
Bone

Figure 5.1. Proposed model for the augmentation of osteoclastogenesis by C1q. Precursor cells of monocyte/macrophage lineage are recruited to the bone. M-CSF and RANKL, produced by the mesenchymal cells/osteoblasts, are essential cytokines required for osteoclastogenesis (1). The binding of M-CSF to M-CSFR induces the expression of RANK (2) and allows increase binding of RANKL to the precursor cells. As the precursor cells mature, they also start to produce and secrete C1q (3). Some of the C1q are deposited on the surface on the bone and the mononucleated osteoclasts/preosteoclasts are "primed" by these immobilized C1q. This increases the expression of M-CSFR and RANK and increase the sensitivity of these cells to M-CSF and RANK (4). Subsequently, the transcription factor NFATc1 – master regulator of osteoclastogenesis – is induced which leads to the upregulation of the osteoclast markers (5). In the mean time, fusion between the mononucleated osteoclasts/preosteoclasts leads to the formation of the multinucleated osteoclasts which eventually become bone-resorbing osteoclasts (6). During the process, the production of C1q is decreased and this may represent an intrinsic regulation of osteoclastogenesis, by preventing excessive priming of the osteoclasts.

Previous studies have indicated the role of complement in osteoclastogenesis (Ignatius *et al.*, 2011; Sato *et al.*, 1993; Tu *et al.*, 2010). Complement activation in the bone microenvironment produced C3a and C5a which enhanced osteoclast differentiation through modulating IL-6 production (Tu *et al.*, 2010). Furthermore, C3a and C5a can induce the production of RANKL by osteoblasts in the presence of IL-1β which also help in osteoclast differentiation (Ignatius *et al.*, 2011). In our assay, we did not use a co-culture system in generating osteoclasts. Furthermore, the serum used for cell culture was HI and complement activation is not likely to occur in our setup. Hence, the effects of complement activation on osteoblasts are not involved in the observed augmentation of osteoclastogenesis by immobilized C1q.

However, cells in the bone microenvironment also synthesize other complement proteins. Bone marrow cells were found to produce Factor B and Factor D (Tu *et al.*, 2010). Complement regulatory proteins such as CD46, CD55 and CD59 were expressed by mesenchymal stem cells, osteoblasts and osteoclasts (Ignatius *et al.*, 2011). Mesenchymal stem cells and osteoblasts also produced C3 and C5 whereas only C3 was detected in osteoclasts (Ignatius *et al.*, 2011). We have also detected the presence of C1r and C1s in osteoclasts at the mRNA levels (data not shown). Although other cell types are not used in

the generation of osteoclasts, we cannot rule out that complement activation is involved in the effects given that we also observed the upregulation of IL-6. However, given that soluble C1q did not exert any effects on osteoclast differentiation (Figure 3.10), we believe that complement activation may not play a significant role in augmenting osteoclastogenesis and the effects observed are mainly due to a direct effect of immobilized C1q.

To rule out the involvement of complement activation in immobilized C1q-enhanced osteoclastogenesis, the presence of complement activation products such as C3a and C5a in the culture media can be measured by ELISA. In addition, antagonists against the receptors for C3a and C5a, which were involved in complement activation-mediated osteoclastogenesis (Tu *et al.*, 2010), can be used to determine the role of complement activation in our system.

During the study, commercially available C1q is often used in many experiments. The purity of C1q used for the study was determined for by silver staining (Appendix 2). No contaminants were detected in the C1q used for our assays. Hence, we can conclude that the effects observed are due to C1q.

5.4 Clinical significances of C1q-mediated osteoclastogenesis

5.4.1 Significance in SLE

SLE presents diverse clinical manifestations and musculoskeletal symptoms are one of them (Rahman and Isenberg, 2008). Patients with SLE reported articular pain and have rheumatoid-like deformities (ulnar deviation, tendinopathies and subluxation) (Schwarz *et al.*, 2006). However, unlike other inflammatory arthritis such as rheumatoid and psoriatic arthritis, cartilage degradation and bone resorption are absent from most patients. The joint presentations of SLE patients are similar to that of Jaccoud's arthritis in which joint deformities are found without bone and joint damage. No previous report has shown the role of complement in lupus arthritis or Jaccoud's arthritis. Our results showing C1q augments osteoclastogenesis suggest possible involvement of C1q in these diseases.

Despite the rarity of genetic C1q deficiency, acquired C1q deficiency is commonly observed in SLE patients especially during renal flares (Sinico *et al.*, 2009; Tsirogianni *et al.*, 2009). Acquired C1q deficiency is due to increased consumption of C1q by the presence of immune complexes and anti-C1q autoantibodies (Greisman *et al.*, 1987; Sinico *et al.*, 2009; Tsirogianni *et al.*, 2009). In pediatric SLE patients, they have increased bone loss which is linked to drugs used in the treatment but not increased osteoclastogenesis (Baker-Lepain *et al.*, 2011). Instead, these patients displayed reduced osteoclast development and functions as indicated by decreased serum TRAP and urine N-telopeptide (Baker-Lepain *et al.*, 2011). Furthermore, the severity of pediatric SLE is correlated with decreased level of serum C1q (Wu *et al.*, 2011). These evidence and our results suggest the possibility of C1q being involved in mediating the effects observed in SLE patients. However, additional experiments are required to establish the link between C1q and the non-erosive nature of lupus arthritis or Jaccoud's arthritis.

5.4.2 Significance in other inflammatory diseases

Many inflammatory diseases are associated with bone resorption. These include rheumatoid arthritis and periodontal disease in which bone erosions are observed at the joints and alveolar bone of the jaw respectively (Lacativa and Farias, 2010). During inflammation, many soluble factors are produced which include cytokines and chemokines. These factors mediate the recruitment of other inflammatory cells and the activation of resident cells and newly recruited cells (Feghali and Wright, 1997). For example, TNF-α can mobilize osteoclast precursor cells from the bone marrow in inflammatory arthritis (Li *et al.*, 2004). Cytokines such as TNF-α, IL-6 and IL-17A promote osteoclastogenesis through direct effects on osteoclast precursor cells or indirect effects by inducing the production of other positive regulators of osteoclastogenesis such M-CSF and RANKL in osteoblasts and fibroblasts (McInnes and Schett, 2007). Not only does the cytokines promote osteoclastogenesis, some cytokines like IL-1α, IL-1β and RANKL also cause osteoclast activation.

Our results have shown that IFNγ and LPS can regulate production of C1q in osteoclasts (Figure 3.7). Furthermore, the production of C1q in other cell types can be regulated by cytokines, drugs and TLR ligands (Lu *et al.*, 2008). This indicates cytokines such as TNF-α, IL-1β and IL-17A present in inflammatory conditions may potentially regulate the production of C1q by osteoclasts, enhance osteoclast differentiation and help in the pathogenesis of the disease. Additional experimental work is required to investigate the effects of various stimulants on C1q production in osteoclasts and their respective role in osteoclastogenesis.

In addition, activated T cells express RANKL which promotes osteoclastogenesis and mediates bone loss in arthritis (Horwood *et al.*, 1999; Kong *et al.*, 1999). T cells also produce IFNγ during inflammatory conditions (Schoenborn and Wilson, 2007). IFNγ produced by T cells is shown to inhibit osteoclastogenesis by interfering with the RANK-RANKL signaling pathway (Takayanagi *et al.*, 2000). Thus, during health, the positive and negative

regulators are balanced and prevent excessive osteoclast formation. However, in diseases, the balance is shifted in favor of the positive regulators, causing aberrant formation and activation of osteoclasts and results in bone erosion.

We showed that IFNγ is able to stimulate the production of C1q in osteoclasts by a large magnitude (Figure 3.7). The increased C1q production was observed in both preosteoclasts and mature osteoclasts (Figure 3.7C) and this could potentially break the intrinsic regulation of osteoclastogenesis by the upregulation of C1q in mature osteoclasts. Thus, IFNγ-induced C1q may be one of the factors that tilt the balance, augment osteoclastogenesis and cause bone erosion during inflammatory arthritis. This provides another dimension in the regulation of osteoclastogenesis by T cells during health and disease though more experiments are needed to establish these links.

Similar to SLE, C1q deficiency and suppression are linked to the development of rheumatoid arthritis (Mizuno, 2006). However, C1q also acts as a double edged sword in rheumatoid arthritis by activating the complement system. Complement activation can induce and augment inflammation (Walport, 2001) and is involved in the pathogenesis of rheumatoid arthritis. Complement proteins and its activation products are detected in the synovial fluid and synovial membrane of patients (Gulati *et al.*, 1994; Morgan *et al.*, 1988; Neumann *et al.*, 2002). Furthermore, products of complement activation are elevated in the synovial fluid of patients with rheumatoid arthritis (Brodeur *et al.*, 1991; Morgan *et al.*, 1988).

Previously, both classical and alternative pathways are involved in the pathogenesis in collagen-induced arthritis in mice (Hietala *et al.*, 2002; Hietala *et al.*, 2004). Collagen-induced arthritis is an experimental animal model for human rheumatoid arthritis and shares several pathological features such as synovial hyperplasia, infiltration of mononuclear cells, pannus formation, cartilage degradation and bone erosion (Brand *et al.*, 2007). Furthermore, C3a and C5a produced by complement activation through the alternative pathway promote osteoclast differentiation (Tu *et al.*, 2010). C1q production by osteoclasts in the bone microenvironment may be involved in maintaining bone

homeostasis by removing apoptotic cells or immune complexes. However, in inflammatory diseases such as rheumatoid arthritis, excessive osteoclast formation and local synthesis of C1q by osteoclasts may exacerbate the disease by inducing more complement activation through the classical pathway. This may lead to the increased formation of osteoclasts and also result in more tissue injuries. The potential effects of C1q in inflammatory diseases that are associated with bone resorption remain to be studied.

5.5 Anti-adhesive effects of immobilized C1q

Cells express many cell surface adhesion receptors such integrins which interact with the extracellular matrix (ECM) (Arthur *et al.*, 2002). Integrins control cellular adhesion and shape which are critical factors for cell survival (Stupack and Cheresh, 2002). Adhesion to the ECM involves adhesive structures such nascent adhesion, focal complex and focal adhesion that differ in localization, size, shape, protein composition and dynamics (Parsons *et al.*, 2010; Valdembri and Serini, 2012). During the formation of cell-ECM adhesions, cells display morphological changes which include rearrangement of actin cytoskeleton mediated by Rho family GTPases. Several stages are involved in the adhesion of cells to the ECM (Frame and Norman, 2008; Huveneers and Danen, 2009). Upon contact to the ECM, early spreading of the cells and assembly of focal adhesions occur. This is accompanied with the activation of Rac1 and Cdc42 which enhances actin-mediated protrusive activities at sites of adhesion. RhoA is also inhibited to suppress actomyosin contractibility. Subsequently, at the later stages, the activity levels of RhoA increase with inhibition of the activities of Rac1 and Cdc42, leading to formation of stress fibres, maturation of focal adhesions and actomyosin contractibility. Contacts between actomyosin contraction and ECM slow actin rearward movement and favor spreading of microtubules, leading to adhesion maturation and fully spread cells. During the process, focal adhesion kinase (FAK) is activated and it is involved in controlling survival, proliferation, apoptosis and migration of the cells (Abbi and Guan, 2002).

Cells of the myeloid lineage such as macrophages and DCs have small and highly dynamic adhesions that facilitate rapid movement on ECM substrates (Parsons *et al.*, 2010). On the other hand, contractile cells such as endothelial cells and fibroblasts have more prominent and stable adhesions. Thus, this accounts for the morphologies of the different cell types. Our results showed that myeloid cell types, primary cells or cell lines, generally exhibited rounded morphology whereas contractile cell types (epithelial cells, fibroblasts and myoblasts) were fully spread and extended (Figure 4.1). All the cell types displayed varying changes in their morphologies in the presence of

immobilized C1q. In general, cells became more rounded and tended to cluster together (Figure 4.1). However, some cell types still displayed certain extent of spreading and these were mainly the non-immune cell types (Figure 4.1).

It is shown previously that soluble C1q can affect DC morphology when used for the generation of DC from monocytes (Castellano *et al.*, 2007). Hence, the effects of soluble C1q on were also tested in our system. In contrast to the distinct effects observed for immobilized C1q, soluble C1q did not affect the morphologies of the cells (Figure 4.2). We have also shown that immobilized but not soluble C1q enhanced osteoclast differentiation (Figure 3.10). This further strengthens the idea that the morphological changes induced by immobilized C1q are linked to the regulatory effects that C1q has on monocyte development.

As cell adhesion is associated with cell morphology, we also investigated the effects of immobilized C1q on the adhesion properties of different cell types. Most of the cell types exhibited reduced adhesion in the presence of immobilized C1q (Figure 4.3). In addition, there is a correlation between morphological changes and cell adhesion in which cells grown in C1q-coated wells were less adherent and they displayed more morphological changes as compared to control cells (Figures 4.1 and 4.3). This is reflective of the mechanisms mediating cell adhesion and cell spreading (Huveneers and Danen, 2009). Thus, the results suggest that the effects of immobilized C1q are ubiquitous and not restricted to monocytes or monocyte-derived cells. A common receptor among these cell types may be involved in mediating these effects. This also reflects the ability of C1q to regulate the functions of different cell types (Lu *et al.*, 2008; Nayak *et al.*, 2010).

Detachment of cells from the ECM will induce apoptosis, a process known as anoikis (Frisch and Screaton, 2001). Integrin-mediated adhesion to the ECM provides cell survival signals via FAK (Abbi and Guan, 2002; Frisch and Screaton, 2001). Furthermore, cellular adhesion is required for the proliferation of the cells. In the presence of immobilized C1q, cells are generally non-adherent which may lead to their apoptosis. To overcome this

problem, they adhere to each other through cell-cell adhesions and form cell clusters. Cadherins are molecules that mediate intercellular adhesion by calcium-dependent homophilic interactions (Fukata and Kaibuchi, 2001). Although cadherin-mediated cell-cell adhesions have a distinct phosphorylation pattern as compared to integrin-ECM adhesion, many common proteins and signaling pathways are activated in both cell-cell and cell-ECM adhesion (Westhoff and Fulda, 2009).

The formation of cell clusters in the presence of immobilized C1q is similar to one of the mechanisms in which tumor cells prevent anoikis upon ECM detachment. Tumor cells show reduced apoptosis when they form aggregates after detachment from ECM (Bates *et al.*, 2000; Westhoff and Fulda, 2009). The exact mechanism is not elucidated but may potentially involve the PI3K/Akt signaling pathway and pathways regulating cell cycle progression, pathways which are also activated by integrin-mediated cellular adhesion. Although the effects of immobilized C1q were observed in the cancer cell lines used, primary cells such as human monocytes, macrophages and DCs also showed similar effects. Thus, forming cell clusters may be a common mechanism to prevent anoikis and possibly account for our results though more studies are required to establish the links.

An interesting point for the cell clusters observed is that they resemble spheroids. Spheroids are generated from spheroid culture which is a type of three-dimensional (3D) cell culture system (Fennema *et al.*, 2013). Cells in 3D culture are different to those in 2D cultures as they have gene expression profiles that are reflective of endogenous expression profiles. Spheroid models of embryonic stem cells and tumor cells have been used to study cellular differentiation, cell-cell interactions, hypoxia response and therapeutic purposes (Bates *et al.*, 2000; Fennema *et al.*, 2013; Frith *et al.*, 2010; Wang *et al.*, 2009). Many methods are available for the generation of spheroids and the main principle is preventing the adherence of cells to the substratum (Bates *et al.*, 2000; Lin and Chang, 2008). This is similar to our setup as immobilized C1q acts as the anti-adhesive layer which prevents the adherence of cells, leading to the formation of spheroid-like cell clusters.

Spheroid human mesenchymal stem cells secrete more anti-inflammatory proteins than cells grown as adherent monolayer (Bartosh *et al.*, 2010). These cells also showed decreased activation of macrophages *in vitro* and reduced inflammation *in vivo*. Furthermore, DC generated on immobilized C1q exhibit similar spheroid formation and are tolerogenic (Teh *et al.*, 2011). Thus, aggregation of cells or formation of spheroids may be important in generating an anti-inflammatory response. The changes in signaling patterns from integrin-mediated to cadherin-mediated could be responsible for the anti-inflammatory effects observed though the exact mechanism remains to be investigated.

5.6 Known C1q receptors are not likely to be involved in immobilized C1q-induced effects

Many proteins are found to bind to C1q and mediate its functions in different cells (Lu *et al.*, 2008). These C1q receptors include $\alpha_2\beta_1$, CD35, CD91, CD93 and gC1qR. We have shown that immobilized C1q is able to affect the morphologies and adhesion of various cell types to varying extents (Figures 4.1 and 4.3). Furthermore, the effects of immobilized C1q are specific and immediate (Figures 4.4 and 4.5). This implies that the cells used may express a common C1q receptor which mediates the effects of immobilized C1q. Depending on the cell type used, the kinetics for the effects of immobilized C1q differed (Figures 4.5A and 4.8A). RAW264.7 cells displayed aggregation after 1 hour of incubation and the extent of aggregation became more obvious at later time points (Figure 4.5A). For HEK293T cells, cell aggregation was only obvious after 4 hours of incubation (Figure 4.8A). However, both cell lines exhibited similar morphological changes after incubating for 24 hours (Figures 4.5A and 4.8A). Thus, the results suggest that the amount of C1q receptor present on the cells may affect the kinetics of immobilized C1q. This may also account for the different extent in morphological changes and decrease in adhesion observed for different cell types (Figures 4.1 and 4.3).

A comparison between different human cell lines was then performed to establish if the expression of known C1q receptors differs which can explain the results observed. However, detection of the receptors at both the mRNA and protein levels did not yield any significant association between the receptor levels and the observed morphological changes (Figures 4.6A and 4.6B). Next, antibodies specific to C1q receptors were used to determine if these receptors were involved in mediating the morphological changes by C1q. Blocking of the receptors did not inhibit the effects of immobilized C1q (Figure 4.6C). An issue is that the nature of these antibodies is not fully characterized and it is not known if they can function as blocking antibodies. However, we believe that binding of the antibodies should provide structural hindrances for the interactions between the receptor and C1q. Furthermore,

these antibodies are not likely to provide activating signals similar to that of immobilized C1q as no morphological changes were observed in control cells incubated with these antibodies (Figure 4.6C).

The whole C1q molecule consists of two major domains which are the globular heads and collagen tail (Figure 4.7A) (Reid and Porter, 1976). The identified C1q receptors are known to bind to either the globular head or the collagen tail. Thus, the two domains were generated which may allow the screening of receptors that mediate the effects of immobilized C1q. However, both domains did not affect the morphologies of the cells as compared to cells incubated with immobilized C1q (Figures 4.7C and 4.7D). The results further validate that $\alpha_2\beta_1$, CD35, CD91, CD93 and gC1qR are not involved in immobilized C1q-induced effects as they bind specifically to the globular head or collagen tail of C1q. During the course of the study, new C1q receptors such as RAGE, Fz and LAIR-1 are identified (Ma *et al.*, 2012; Naito *et al.*, 2012; Son *et al.*, 2012). Hence, these receptors or an unknown C1q receptor may mediate the effects of immobilized C1q. More experimental work is required to identify the receptor involved.

5.7 Augmentation of morphological changes by anti-C1q antibodies

Anti-C1q autoantibodies are present in many SLE patients and they are associated with lupus nephritis (Sinico *et al.*, 2009; Tsirogianni *et al.*, 2009). Increases in anti-C1q autoantibodies have been associated with renal flares. Furthermore, lupus nephritis does not develop when anti-C1q autoantibodies are absent in patients (Trendelenburg *et al.*, 1999). These autoantibodies mainly target neoepitopes exposed at the collagen tails after immobilization of C1q (Antes *et al.*, 1988; Uwatoko *et al.*, 1987; Wener *et al.*, 1989). Our results suggest that these neoepitopes may be involved in mediating the effects of immobilized C1q given that high amounts of globular heads and collagen tails were not able to induce morphological changes in the cells as compared to that of immobilized intact C1q (Figure 4.7). Hence, commercial anti-C1q antibodies and anti-C1q autoantibodies from patients were used to test the effects of blocking immobilized C1q.

Interestingly, commercial anti-C1q antibodies enhanced the kinetics of immobilized C1q (Figures 4.8A and 4.8B). The augmentation of immobilized C1q-induced effects are likely due to the binding of anti-C1q antibodies to C1q globular heads as the monoclonal anti-C1q antibody is specific for globular heads of C1q. Binding of C1q to immune complexes is not involved in enhancing the kinetics as high salt was used during incubation with anti-C1q antibodies which prevented immune complexes from binding to immobilized C1q (Kohro-Kawata *et al.*, 2002). Furthermore, isotype controls did not affect the kinetics of immobilized C1q. Thus, specific interactions between anti-C1q antibodies and globular heads mediate the rapid changes in morphologies of the cells. In addition, isolated C1q globular did not affect the morphology of the cells (Figure 4.7E) and the addition of anti-C1q antibodies also did not mediate any changes (Appendix 4). Hence, anti-C1q antibodies may increase the exposure of neoepitopes in the collagen tails upon binding to the globular heads of intact C1q. This allows easier access for C1q receptor to the exposed neoepitopes, increases engagements between C1q receptor and immobilized C1q and enhances the speed of morphological changes in the cells.

However, unlike the commercial anti-C1q antibodies, anti-C1q autoantibodies from SLE patients did not modulate the effects of immobilized C1q (Figure 4.9C). Several reasons may potentially explain for the lack of morphological changes for anti-C1q autoantibodies. The morphological changes are likely due to neoepitopes present in immobilized C1q and the enhancement of such changes is due to the increased exposure of such epitopes as discussed earlier. The commercial anti-C1q antibodies are likely to exert these effects via binding to the globular heads of C1q. Most of anti-C1q autoantibodies recognize and bind to the C1q collagen tails (Antes *et al.*, 1988; Uwatoko *et al.*, 1987; Wener *et al.*, 1989). Despite studies showing the presence of anti-C1q autoantibodies in SLE patients that recognize C1q globular heads (Tan *et al.*, 2009; Tsacheva *et al.*, 2007), the prevalence of such antibodies is low and similar to that of control patients (Tan *et al.*, 2009). Thus, it is not likely to observe augmentation of cellular morphological changes in the presence of anti-C1q autoantibodies.

A recent study has identified two linear epitopes in which a specific anti-C1q Fab can recognize but only one epitope could bind the Fab in ELISA (Vanhecke *et al.*, 2012). Furthermore, anti-C1q Fabs are able to recognize different regions of the C1q collagen tails (Schaller *et al.*, 2009). These studies suggest the diversity of neoepitopes present in immobilized C1q. We also showed that purified collagen tails did not induce changes in cellular morphologies (Figure 4.7C). As anti-C1q autoantibodies can bind to C1q collagen tail-based ELISA (Uwatoko *et al.*, 1987; Wener *et al.*, 1989), the results suggests that neoepitopes recognized by anti-C1q autoantibodies may not be involved in mediating the effects of immobilized C1q. Thus, some new and uncharacterized neoepitopes which are not recognized by anti-C1q autoantibodies could be possibly mediating the morphological changes observed.

On the contrary, neoepitopes recognized by anti-C1q autoantibodies may mediate the effects of immobilized C1q. Given the range of neoepitopes recognized by anti-C1q autoantibodies, the amount of autoantibodies present in the samples may be too low to block the neoepitopes responsible for

immobilized C1q-induced morphological changes. Commercial anti-C1q antibodies are enriched in antibodies against C1q whereas anti-C1q autoantibodies are part of the antibody repertoire of the SLE patients. To enrich the anti-C1q autoantibodies, IgG precipitation was carried out and the results showed concentration of the samples as compared to original samples (Figure 4.9B) However, these concentrated antibodies did not show saturation in our ELISA assay even when high amounts were used whereas both goat and mouse anti-C1q antibodies were able to reach saturation easily (Appendix 5).

Although no morphological changes were observed in cells incubated in collagen tails-coated wells (Figure 4.7C), neoepitopes recognized by anti-C1q autoantibodies may still mediate the effects of immobilized C1q. C1q collagen tail-based ELISA normally has lower readings than intact C1q-based ELISA (Trinder *et al.*, 1996; Uwatoko *et al.*, 1987). This may indicate some of the neoepitopes recognized by anti-C1q autoantibodies may be masked in immobilized C1q collagen tails and unable to affect the morphologies of the cells. These masked epitopes may be responsible for the effects of immobilized C1q and are further exposed by commercial anti-C1q antibodies such that enhanced kinetics of immobilized C1q was observed. More experiments are required to establish the links between anti-C1q autoantibodies and the effects of immobilized C1q.

5.8 Future work

5.8.1 Determine the role of C1q in osteoclastogenesis *in vivo*

In our study, we have shown that immobilized C1q augments osteoclastogenesis. Furthermore, as discussed in 5.4, C1q may play a role in the pathogenesis of several diseases such as Jaccoud's arthritis and rheumatoid arthritis. Thus, it is important to establish the role of C1q in osteoclastogenesis *in vivo*.

Previously, C1q-deficient mice were generated and these mice displayed highly similar phenotypes as SLE patients (Botto *et al.*, 1998). Using this mouse model, the bones and skeletal structure could be examined to determine if there are any defects in the absence of C1q. Subsequently, the bone marrow cells could be used to generate osteoclasts. This will allow us to determine if lack of C1q affects the differentiation of these precursor cells to osteoclasts. The findings will further strengthen our data that C1q regulates osteoclastogenesis and also account for any defects in the bones if observed.

Other than osteoclasts, both osteoblasts and osteocytes are essential in maintaining bone homeostasis (Nakashima *et al.*, 2012). Furthermore, these two cell types also regulate osteoclastogenesis by producing M-CSF and RANKL. Given that C1q is present in the bone microenvironment, the effects of C1q on osteoblasts and osteocytes can be investigated using the C1q-deficient mouse model. This will increase the understanding the role of C1q in the bone microenvironment and how it affects bone remodeling in general.

For Jaccoud's arthritis, the absence of bone and joint damage may be due to the decreased levels of C1q in SLE patients, leading to inhibition of osteoclast formation. The C1q-deficient mouse model may provide insights to the pathogenesis of the disease and establish the link between C1q and the non-erosive nature of Jaccoud's arthritis. The mouse model can also be used to increase the understanding of the role of C1q-induced osteoclastogenesis in diseases associated with bone resorption.

5.8.2 Investigate the effects of IFNα and C1q on osteoclast differentiation

Other than the role of C1q in SLE, type I IFN have been shown to play a critical role in the pathogenesis of SLE (Banchereau and Pascual, 2006). The severity of the disease was found to correlate with serum levels of IFNα (Hooks *et al.*, 1979). Furthermore, PBMCs of SLE patients have been shown to have the IFN transcriptome in which IFN-regulated genes are highly upregulated (Baechler *et al.*, 2003; Bennett *et al.*, 2003). Type I IFN is also shown to inhibit osteoclastogenesis (Coelho *et al.*, 2005; Takayanagi *et al.*, 2002). A recent study has shown that IFNα in SLE directs monocytes to differentiate into myeloid DC instead of osteoclasts, leading to decreased bone erosion observed in Jaccoud's arthritis (Mensah *et al.*, 2010).

Understanding the interplay between osteoclast-derived C1q and type I IFN may further our knowledge on osteoclast differentiation in both normal and pathological conditions. For example, osteoclasts incubated with immobilized C1q could be more resistant to Type I IFN inhibition. In addition, osteoclast-derived C1q may inhibit IFNα production by plasmacytoid DC, the main producers of IFNα, in the bone microenvironment. On the other hand, Type I IFN may inhibit osteoclast differentiation by suppressing production of C1q by preosteoclasts. These possibilities required more experimental work to be done to further understand the roles of C1q and IFNα in osteoclastogenesis.

5.8.3 Identify the C1q receptor involved in mediating the anti-adhesive effects of immobilized C1q

Beside its role in complement activation, C1q is involved in diverse biological processes (Lu *et al.*, 2008; Nayak *et al.*, 2010). In particular, our study and previous studies have shown the significance of C1q in modulating the development of monocytes (Castellano *et al.*, 2007; Fraser *et al.*, 2009; Teh *et al.*, 2011). Immobilized C1q limits the immune response of monocytes, macrophages and DCs to LPS (Fraser *et al.*, 2009). Furthermore, tolerogenic DCs are generated from monocytes in the presence of C1q which reduce T cell

response (Castellano *et al.*, 2007; Teh *et al.*, 2011). Our results have also indicated that C1q is able to modulate osteoclast differentiation from monocytes. Thus, we set out to determine the C1q receptor responsible for these effects induced by immobilized C1q. However, despite our attempts, the C1q receptor remains elusive.

New C1q receptors such as RAGE, Fz and LAIR-1 are identified during the course of study (Ma *et al.*, 2012; Naito *et al.*, 2012; Son *et al.*, 2012). These receptors are not considered during the start of our study and may potentially be involved in modulating the effects of immobilized C1q on monocytes. Hence, these receptors will be further studied to determine if they are involved in modulating monocyte development by C1q. Furthermore, knockdown of specific C1q receptors can be employed to determine their role in mediating the effects of immobilized C1q.

An unknown C1q receptor may be responsible for the regulation of monocyte development by C1q. To identify this receptor, cell surface proteins can be biotinylated and concentrated with strepavidin beads (Weekes *et al.*, 2010). Concentrated surface proteins are then incubated with C1q-coated beads to isolate potential receptors. The identities of these pull-down receptors will be determined and the involvement of these receptors in immobilized C1q-induced effects will be validated using knockdown experiments. As commercial anti-C1q antibodies enhanced the kinetics of immobilized C1q (Figures 4.8A and 4.8B), C1q-coated beads can be incubated with these antibodies first before pull-down of the receptors is performed. This may increase the chances of obtaining the unknown C1q receptor. Identification of the receptor will enable understanding of the mechanism involved in C1q regulation of monocyte development.

Chapter 6 – Conclusion

C1q plays many roles in immune and non-immune functions (Lu *et al.*, 2008; Nayak *et al.*, 2010). This is partially achieved through regulating the activities of different cell types. As tissue access to serum C1q may be limited, local synthesis of C1q is essential to regulate cellular functions. Localized C1q can be observed in many tissues and organs such as arteries, articular cartilage, brain, esophagus, liver and placenta (Agostinis *et al.*, 2010; Armbrust *et al.*, 1997; Bobryshev *et al.*, 2010; Bulla *et al.*, 2008; Cao *et al.*, 2003; Schafer *et al.*, 2000; Stevens *et al.*, 2007). Thus, we hypothesized that osteoclasts may be a potential source of C1q given that many similarities exist between osteoclasts and known C1q producers, macrophages and DC.

In our study, we found that cultured osteoclasts produce C1q which was determined by various methods such as qPCR, Western blot, flow cytometry and ELISA. The results were validated using human femoral bone samples and immunohistochemistry which showed the presence of C1q in endogenous osteoclasts. Similar to macrophages and DC, the production of C1q can be regulated by different stimulants. A remarkable increase in C1q was observed when osteoclasts were stimulated with IFNγ and this may be potentially involved in the pathogenesis of inflammatory diseases. Furthermore, immobilized C1q increased the expression of osteoclast markers and suggests a potential role of C1q in osteoclastogenesis. However, the *in vivo* role of C1q in regulating osteoclast differentiation and pathogenesis of osteoclast-related diseases remain to be investigated.

Previous studies have shown tolerogenic DCs were generated from monocytes in the presence of C1q (Castellano *et al.*, 2007; Teh *et al.*, 2011). Our study also showed that C1q regulated osteoclast differentiation from monocytes. Distinct morphological changes were also observed in DCs when C1q was used during differentiation (Castellano *et al.*, 2007; Teh *et al.*, 2011). Thus, the role of C1q on monocyte development may involve the accompanying morphological changes observed. Understanding how C1q affects cellular morphology will provide insights on the possible mechanisms

involved in the regulation of monocyte development by C1q. We hypothesized that a common receptor is present on monocytes and DCs that interacts with immobilized C1q and responsible for the effects of immobilized C1q.

We found that immobilized C1q affected the morphologies and adhesive properties of all the primary cells and cell lines used. However, soluble C1q did not mediate any morphological changes in the cells. This reflects the results obtained with osteoclasts in which soluble C1q did not affect osteoclast differentiation and strengthen the link between C1q-induced morphological changes and regulation of monocyte development by C1q. However, known C1q receptors such as $\alpha_2\beta_1$, CD35, CD91, CD93 and gC1qR are not involved in mediating the effects of immobilized C1q. Although we did not identify the C1q receptor involved in our observations, we determined that intact C1q was required for the effects of immobilized C1q. Furthermore, commercial anti-C1q antibodies were able to enhance the effects of immobilized C1q by increasing the speed of morphological changes. The results imply that neoepitopes exposed during C1q immobilization may be involved in mediating the effects observed and warrants further investigation.

In conclusion, local synthesis of C1q plays an important role in regulating cellular functions. Although C1q may be produced in low amounts in the microenvironment, the concentration may be high enough to have a significant role in regulating cellular functions. This is especially critical given that access to serum C1q may be limited in the absence of inflammation. Furthermore, local synthesis of C1q may contribute to the pathogenesis of diseases in association with bone resorption. Given that many receptors are known to bind C1q, it is not surprising that activities of many cell types are regulated by C1q. By understanding the mechanisms in which C1q affects monocyte development, the findings can be extended to other cell types potentially affected by similar mechanisms or vice versa. Thus, more insights of the role C1q plays in different diseases such as SLE, rheumatoid arthritis and Alzheimer's disease can also be obtained.

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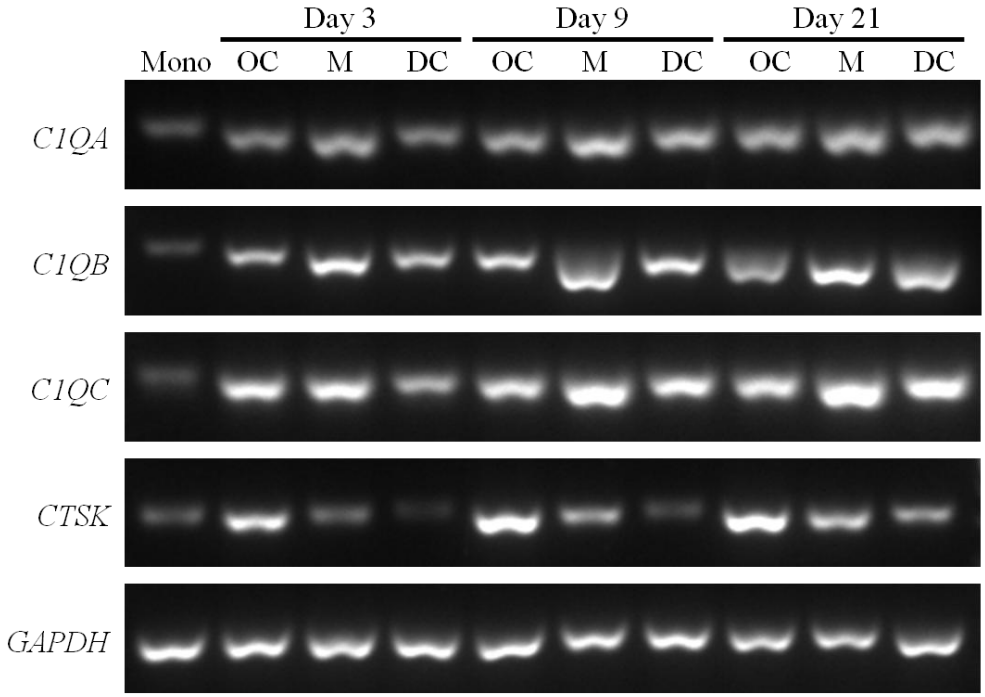
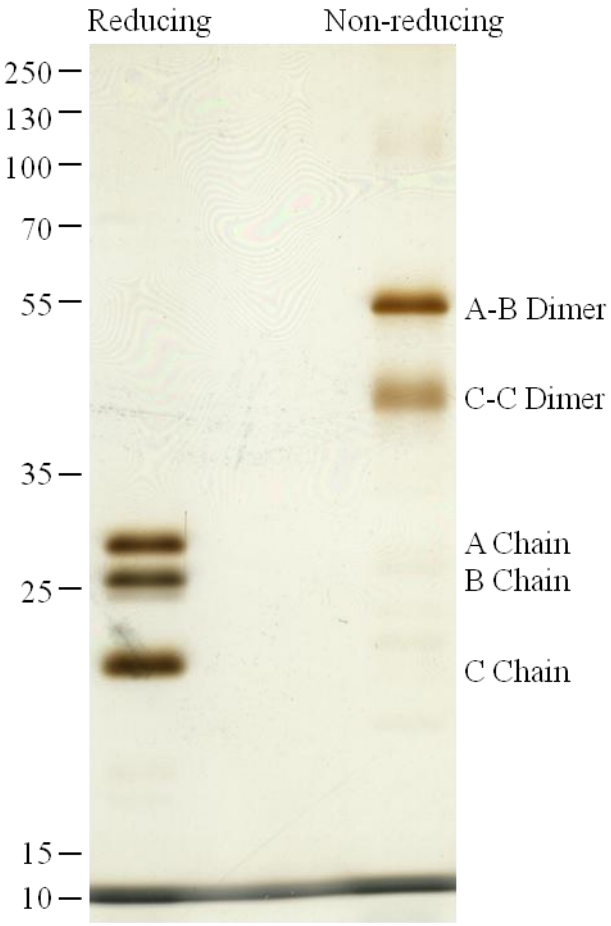
Appendices

Appendix 1. Expression of C1q genes by human primary cells.



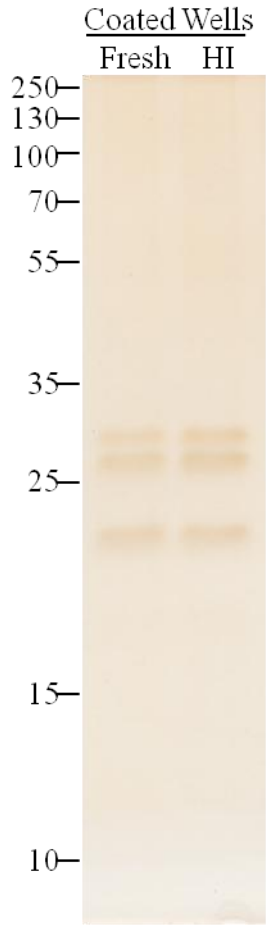
RT-PCR was performed to determined the expression of C1q (*C1QA*, *C1QB* and *C1QC*) genes in monocytes (Mono), osteoclasts (OC), macrophages (M) and DC. *CTSK* and *GAPDH* were used as osteoclast marker and endogenous control respectively.

Appendix 2. Purity of C1q used for the study.



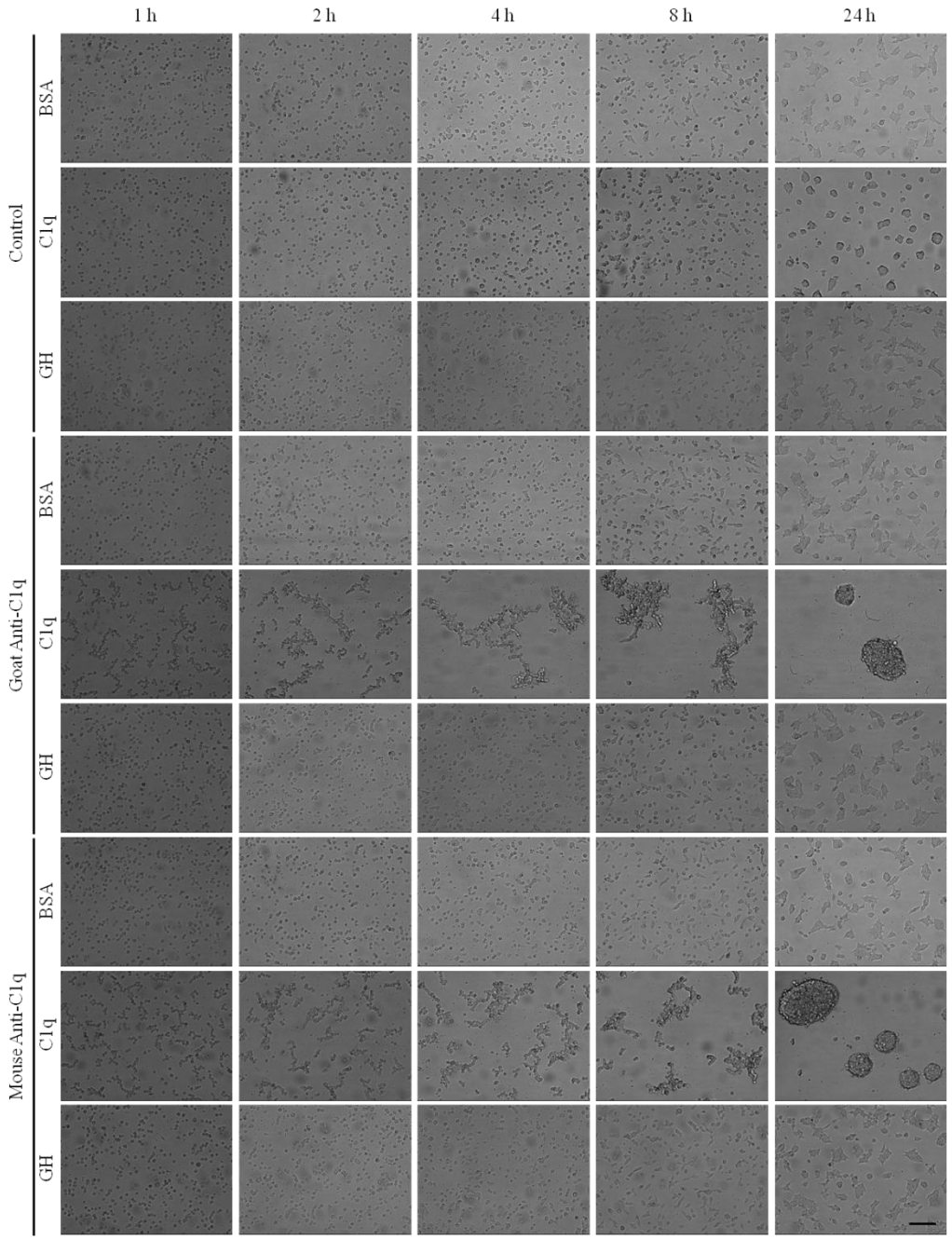
The purity of commercial C1q used for the study was determined by silver staining under reducing and non-reducing conditions. The three chains (A, B and C chains) are observed under reducing condition. Without reduction, the three chains exist as A-B heterodimer and C-C homodimer due to the disulphide bonds joining the chains.

Appendix 3. Quantification of the amounts of coated C1q for fresh and HI C1q.



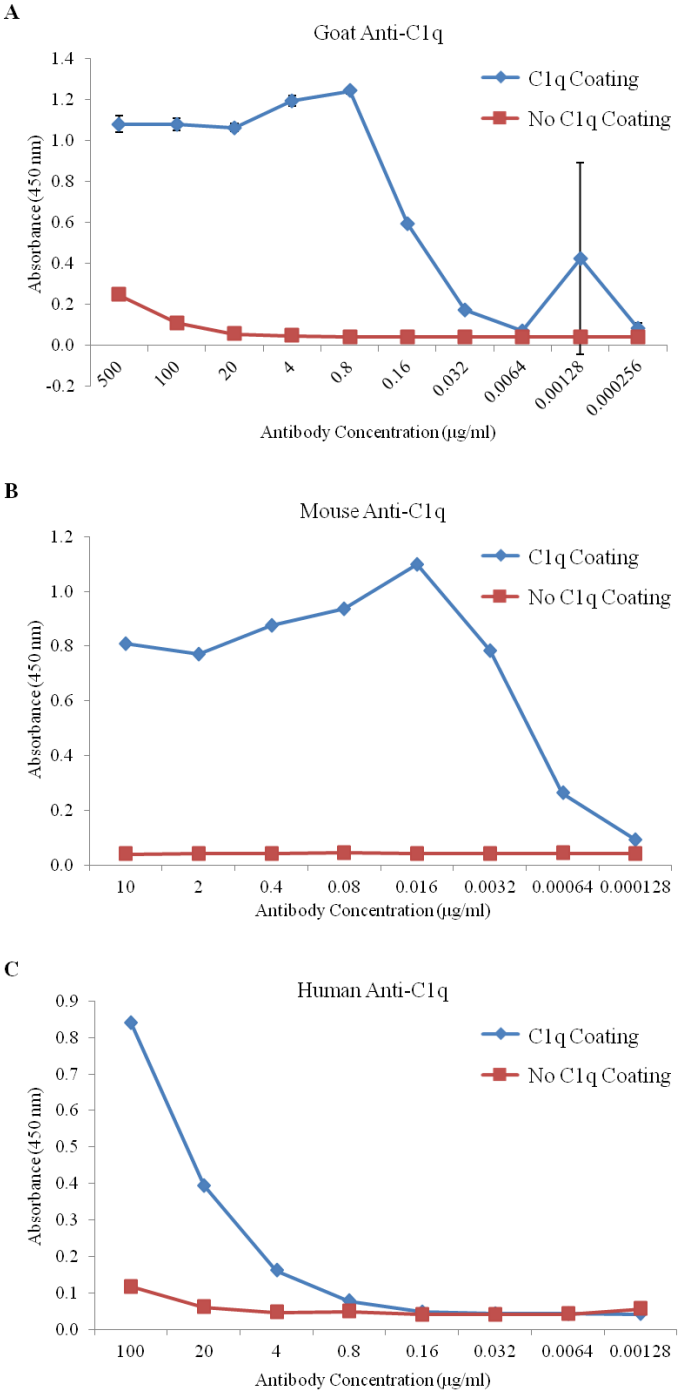
C1q was HI at 56^{o}C for 30 minutes before coating was performed with both fresh and HI C1q. The tissue culture plate was washed thrice with 1X PBS after coating. Coated C1q, fresh and HI, was then eluted from the wells with 2% SDS. The amounts of coated C1q were then determined by silver staining.

Appendix 4. Effects of commercial anti-C1q antibodies and C1q globular heads (GH) on cellular morphologies.



Tissue culture plates were first coated with BSA, intact C1q or C1q globular heads (GH). The plates were then blocked and subsequently incubated with various antibodies including goat anti-C1q and mouse anti-C1q. Cells were then added to the wells and microscopic images were taken at various time points. Scale bar represents 25 μm. The results are representative of three independent experiments.

Appendix 5. Dose titration of anti-C1q antibodies.



The amount of anti-C1q antibodies in the commercial anti-C1q antibodies and human anti-C1q autoantibodies were determined by ELISA. Wells were either uncoated or coated with C1q before anti-C1q antibodies – goat anti-C1q **(A)**, mouse anti-C1q **(B)** and human anti-C1q **(C)** – were added in the presence of high salt (1M NaCl) to prevent the binding of immune complexes to immobilized C1q. The results are representative of two independent experiments.