The Role of Complement In Tumour Formation: Friend Or Foe?

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Summary

The aims of this thesis were to investigate and build on recent reports implicating the complement (C) system in tumour growth and progression. The respective contributions of specific C components and regulators to this effect were assessed by the use of two tumour induction protocols.

Firstly, the chemical carcinogen 3-methylcholanthrene (3-MCA) was used to induce tumours in wild type mice and in animals deficient in C1q, C3 or double deficient in CD55 and CD59. Deficiency in the classical pathway of activation ($C1q^{-/-}$) or in the central component ($C3^{-/-}$) conferred a statistically significant protective effect against 3-MCA-induced tumourigenesis, suggesting that a fully functional C system was important for promotion of tumour progression *in vivo*. Additionally, a protective effect was observed in $CD55^{-/-}.CD59^{-/-}$ mice indicating an important role for C-regulatory proteins (CRegs) in tumour progression.

In the second approach, novel fibrosarcoma lines were generated from 3-MCA-induced tumours. WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ lines were cloned and tested for specific characteristics including CReg expression, synthesis of C3 and susceptibility to C-attack. Few differences were observed between lines of different genotypes though expression of terminal pathway regulator CD59 was observed in $C3^{-/-}$ lines only. Re-inoculation of WT and $C3^{-/-}$ lines showed no differences between the groups with comparable tumour incidence and growth rates observed.

Additionally, the effect of C on progression of a pre-characterised WT fibrosarcoma line was tested by inoculation into WT and C-deficient mice. Tumours inoculated into $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice were shown to exhibit comparable growth characteristics to those in WT controls. However, depletion of the terminal pathway component C5 using a monoclonal antibody (mAb) was shown to inhibit tumour progression. Treatment of mice with anti-C5 mAb conferred a statistically significant protective effect to these mice and suggests a role for C in driving tumour pathology.

In conclusion, work in this thesis demonstrates an important pro-tumour role for C whereby activation of C can result in enhanced tumour progression. Additionally, expression of the CRegs CD55 and CD59 on host cells rather than tumour cells contributes to tumour proliferation. A pro-tumour role for C is contrary to current dogma but supports an alternative hypothesis whereby cell activating effects may provide a selective advantage to tumour cells following C-activation.

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Abbreviations

Ab	Antibody
AC	Apoptotic Cell
ADCC	Antibody Dependent Cellular Cytotoxicity
AP	Alternative Pathway
AT	Anaphylatoxin
BSA	Bovine Serum Albumin
C	Complement
C4bp	C4 binding protein
Calcein-AM	Calcein Acetoxymethyl ester
CDC	Complement Dependent Cytotoxicity
CHO	Chinese Hamster Ovary
CLI	Complement Lysis Inhibitor
CP	Classical Pathway
CR	Complement Receptor
CReg	Complement Regulator
Crry	Complement Receptor Related gene Y
DAF	Decay Accelerating Factor
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
ECM	Extra-Cellular Matrix
ELISA	Enzyme linked immunosorbent assay
fB/fD/fH/fI	Factor B/D/H/I
iC3b	Inactive C3b
Ig	Immunoglobulin
IL-2	Interleukin-2
IP	Intra-peritoneal
Kmax	Maximal killing
mAb	Monoclonal Antibody
MAC	Membrane Attack Complex
MASP	Mannose Associated Serine Protease
MBL	Mannose Binding Lectin
MCP	Membrane Co-factor Protein
3-MCA	3-Methylcholanthrene
MDSC	Myeloid Derived Suppressor Cell
MHC	Major Histocompatibility Complex
NK Cell	Natural Killer Cell
pAb	Polyclonal Antibody
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffered Saline
PKC	Protein Kinase C
PI	Propidium Iodide
PNH	Paroxysmal Nocturnal Haemoglobinuria
qPCR	Quantitative Polymerase Chain Reaction

RCA	Regulators of Complement Activation
ROS	Reactive Oxygen Species
SC	Sub-cutaneous
SCR	Short Concensus Repeat
siRNA	Small Interfering Ribonucleic Acid
SLE	Systemic Lupus Erythematosus
s/n	Supernatent
TCR	T-cell receptor
TReg	T-Regulatory Cell
TGF-β	Tumour Growth Factor-β
TNF-α	Tumour Necrosis Factor-α
WT	Wild Type

1. INTRODUCTION

The aims of the work described in this thesis were to investigate the relationship between tumours and the complement (C) system. This system forms a crucial branch of innate immunity with numerous roles including host defence against infection, clearance of immune complexes and contribution to optimal antibody responses. C is known to target and kill cells and it would seem logical to assume that C poses an obstacle against tumour cell survival and proliferation. However, the relationship between C and tumours remains poorly understood and an alternative hypothesis is emerging suggesting that C may actually elicit pro-tumour effects and contribute to enhanced tumour cell survival and proliferation. Whilst this hypothesis remains unproven and appears to contradict dogma, nucleated cells are known to be resistant to lysis by C and are able to withstand C activation and deposition. Various stimulatory effects have been attributed to C activation and deposition on cells which are not lysed. Therefore, it has been suggested that tumour cells may be able to 'harness' the C system and use it to their advantage. In this way, C activation could promote tumour growth and survival rather than contribute to anti-tumour immune responses.

A detailed knowledge of the C system is important to identify key steps and their effects in relation to tumour cells. Three C activation pathways exist and are shown in Figure 1.1:

1.1 The Complement system: Overview

The C system comprises a family of plasma proteins that interact via a cascade mechanism. A crucial effector mechanism of innate immunity, C also provides an important link between innate and adaptive immune responses. Although initially discovered as a bacteriolytic pathway, C has many wide-ranging effects which do not culminate in lysis of target cells. A plethora of proteins exist which contribute to one or more of the activation pathways, or possess regulatory functions able to control autologous C attack (reviewed in

two parts (Walport, 2001a; Walport, 2001b)). Primarily, C components are synthesized by hepatocytes in the liver and circulate in blood plasma (Alper et al. 1969). However, local C biosynthesis by numerous cell types is also well documented and is known to be significant in tissue homeostasis and immune defence (Morgan and Gasque 1997).

An important characteristic of C is the ability to discriminate between self and non-self cells and limit attack to foreign/invading cells whilst causing minimal damage to host tissue. This is partly achieved by tightly controlled activation mechanisms that prevent the inactive 'zymogen' forms of C proteins from depositing until specifically activated. Three separate activation pathways exist (Figure 1.1), each converging at a shared terminal pathway which leads to deposition of the Membrane Attack Complex (MAC), capable of lysing target cells. Upon activation of any of the pathways, precursor C proteins are enzymatically cleaved causing deposition of active protein fragments and localising attack to the activating target.

The enzymatic nature of these pathways means that a relatively small-scale trigger can be readily amplified to elicit a large-scale and rapid C response.

1.1.1 Classical pathway activation

The classical pathway of C activation is antibody-dependent and provides a link between innate and adaptive immunity. 'Foreign' antigens are recognised by specific antibodies to form immune complexes whereupon the Fc portions of IgM and IgG antibodies can then initiate the classical pathway through interaction with the globular domains of the initiator complex C1 (Sim and Reid 1991). C1 is a large multimeric complex made up of 3 different sized subunits: two C1r, two C1s and a single C1q molecule. The C1q protein comprises 18 polypeptide chains that form 6 globular 'heads' each consisting of 3 independently interacting chains (Reid and Porter 1976).

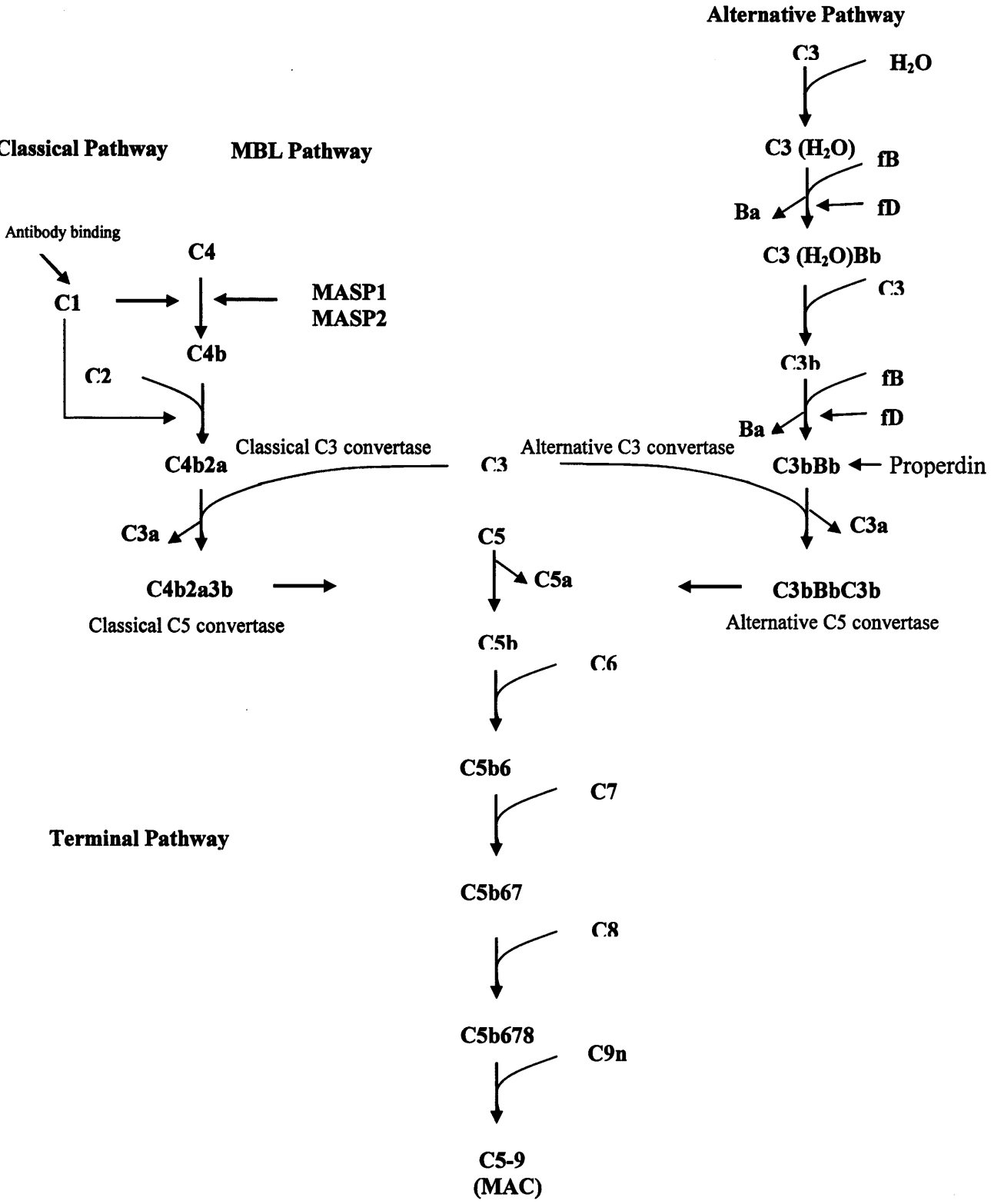


Figure 1.1: The complement system.
Schematic representation of the three activation pathways of complement: classical, alternative and mannose binding lectin. Each pathway converges to a shared terminal pathway leading to insertion of a lytic pore into target cell membranes, the membrane attack complex (MAC). The classical pathway is initiated by either C1q binding to antibodies, or lectin binding to mannose residues. By contrast, the alternative pathway has no specific activation step and instead is centred around spontaneous low level hydrolysis or cleavage by proteases at inflammatory sites.

For activation, 2 of the 6 C1q heads must bind to immunoglobulin Fc (IgG1, IgG2, IgG3 or IgM only) inducing a conformational change in the complex. This change causes a C1r sub-unit to spontaneously activate via autocatalytic cleavage, this activated C1r molecule then cleaves and activates the other C1r sub-unit. The activated C1r molecules then cleave the two C1s sub-units respectively activating serine esterase activity of C1s and enabling cleavage of the next protein in the cascade, C4 (Cooper 1985).

The activated C1s enzyme can cleave numerous C4 molecules making this step capable of rapid amplification of the pathway. The C4 molecule comprises 3 polypeptide chains linked by three disulphide bonds (Schreiber and Müller-Eberhard 1974). Cleavage of this protein yields a weak anaphylatoxin (C4a) (Hugli et al. 1983), which is released, and an unstable C4b intermediate (Dodds and Law 1990). The larger C4b fragment contains a highly labile internal thioester bond which is either rapidly inactivated via aqueous hydrolysis or can bind covalently with amine or hydroxyl groups on the target cell membrane. Surface bound C4b provides a Mg^{2+}-dependent binding site for the next component C2 which binds in an inactive form and releases C2b upon cleavage (Nagasawa and Stroud 1977). This cleavage is also mediated by activated C1s and allows formation of a C4b2a complex known as the 'C3 convertase' of the classical pathway. This enzyme is highly unstable and rapidly loses its function if it cannot immediately bind C3 The short half-life of the C3 convertase and the lability of activated C4b confers a high level of self-regulation upon the pathway and helps to prevent unnecessary activation and autologous attack. Hydrolysis occurs very rapidly in non-bound proteins confining deposition, and therefore attack, to the immediate vicinity of the activation site.

The C3 molecule is common to all three pathways and is the central component of the entire C system. Following classical pathway activation, C3 is cleaved by the serine protease domain of C2a which makes up part of the classical C3 convertase, C4b2a. The C3 molecule comprises 2 protein chains covalently linked via disulphide bonds, while the alpha chain contains a thioester loop buried within the inactive molecule and protected inside a

hydrophobic 'pocket' (de Bruijn and Fey 1985). Upon cleavage of C3 by the C2a portion of the convertase, a small anaphylactic fragment (C3a) is released (Lambris 1988). Like cleavage of C4, once the C3a fragment is removed, the internal thioester loop is exposed and highly susceptible to nucleophilic attack. The unprotected loop is highly reactive, although unstable, and may bind to any nearby antigenic surface. Deposition of the larger fragment C3b with the C4b2a convertase forms a C4b2a3b complex (Kozono et al. 1990), the C5 convertase of the classical pathway and the initiator enzyme of the terminal pathway. Deposited C3b also serves to 'focus' further C attack to the area and it is estimated that approximately 240 C3b molecules are deposited for each activated C1 complex highlighting the amplification potential of classical pathway activation (Ollert et al. 1994).

1.1.2 MBL pathway activation

Another activation pathway closely resembles the classical pathway, sharing many of the components but differing in the initial activation step and thus is not antibody dependent. The mannan-binding lectin (MBL) pathway involves the recognition of mannose and N-acetylglucosamine residues abundant on surfaces of pathogenic micro-organisms via a serum protein known as Mannan Binding Lectin (MBL) (Epstein et al. 1996). This protein, like C1q of the classical pathway, is part of a family known as collectins or collagenous lectins. MBP binds to mannose on bacteria and interacts with two associated serine proteinases MASP-1 and MASP-2, analogous to C1r and C1s of the C1 initiation complex (Matsushita and Fujita 1992). Activation of these enzymes leads to cleavage of C4 and C2 as in the classical pathway and formation of the classical C3 convertase. Another closely related protein, known as MASP-3, is formed by alternative splicing of the MASP-1 gene and has been shown to down-regulate the cleavage capacity of MASP-2 (Dahl et al. 2001).

1.1.3. Alternative pathway activation

C activation can also occur via a third pathway known as the 'alternative pathway', an antibody-independent process based on a 'tickover' mechanism (Nicol and Lachmann 1973). No specific activation step exists in this pathway which instead centres around the spontaneous activation of serum C3 via aqueous hydrolysis of the internal thioester bond of the native molecule. This process is outlined in Figure 1.2. In addition to aqueous hydrolysis, any mechanism by which C3 is cleaved and activated may lead to deposition of C3 fragments and continuation of the alternative pathway. In this way, C3 cleavage enzymes produced locally following inflammatory stimuli can activate the alternative C pathway and lead to C deposition. Cleavage by these mechanisms creates an activated molecule analogous to the C3b cleavage product formed by the classical pathway. Following aqueous hydrolysis, the activated C3(H_2O) reveals a Mg^{2+}-dependent binding site for a 93kDa plasma protein Factor B (fB). This forms a C3(H_2O)B complex susceptible to cleavage by a 26kDa serine proteinase, Factor D (fD) (Volanakis and Narayana 1996). A small fragment of the fB molecule (Ba) is released, while the larger portion is retained in a C3(H_2O)Bb molecule to form an unstable but active C3 convertase. This convertase is able to bind and cleave further C3 molecules via the serine protease domain of fB. This releases the C3a fragment and yields an active C3b fragment. However, as this convertase is in the fluid phase, the vast majority of C3b formed is rapidly inactivated via hydrolysis of the labile thioester bond. As previously stated, this process is spontaneous and occurs ubiquitously in a random and non-specific manner. Continuation of the alternative pathway, or cleavage of C3b to iC3b by inactivating enzymes is dependent on surface properties of the target cell. Such properties include glycosylation patterns, charge and sialation on cell surfaces (Fearon 1979). Surfaces exhibiting low levels of sialic acid are less able to bind the regulatory protein factor H (fH), thus provide an 'activating' surface allowing un-hindered binding of fB and subsequent cleavage by fD to release Ba. This forms the membrane bound C3bBb complex capable of further C3 cleavage.

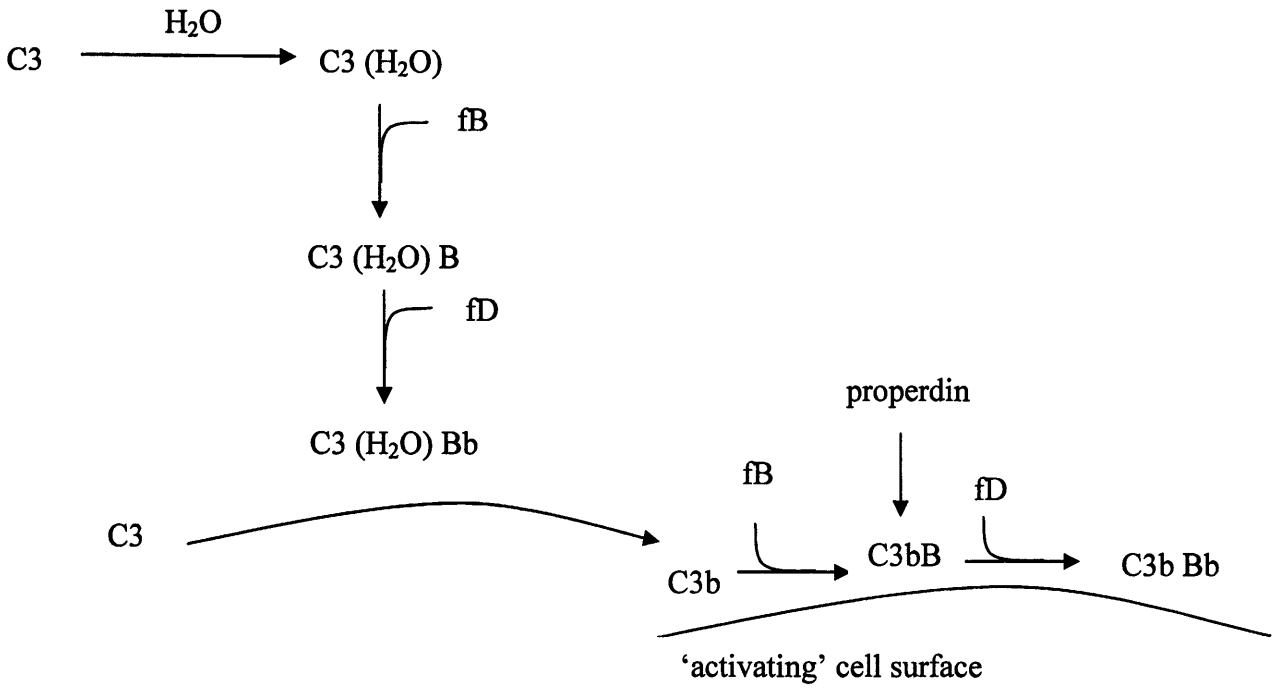


Figure 1.2: The alternative pathway of C activation
Schematic representation of the alternative activation pathway of complement. Spontaneous 'tickover' activation occurs due to hydrolysis of C3 in the fluid phase. An unstable C3 convertase is formed in the fluid phase able to yield C3b fragments capable of depositing on cell membranes.

The C3bBb convertase dissociates rapidly but is effectively stabilised in the presence of properdin, a glycoprotein which acts as a positive regulator on the alternative pathway (Pillemer et al. 1954). This step of the alternative pathway represents an effective amplification mechanism whereby one convertase molecule can cleave numerous further C3 molecules and rapidly amplify the response. As subsequent C3 molecules are cleaved, the larger C3b fragment may associate with C3bBb to form C3bBbC3b which is the alternative pathway C5 convertase and allows initiation of the terminal pathway. A key feature of the alternative pathway is that cleavage of C3 yields a C3b molecule capable of forming another C3 convertase enzyme (through interaction with fB). In this way, the amplification capacity of the alternative pathway is not restricted only to alternative pathway activation and will

effectively amplify activation following initial deposition of C3b via classical, alternative or MBL pathways.

1.1.4 Terminal pathway & MAC formation

Cleavage of C5 by either the classical (C4b2a3b) or alternative (C3bBbC3b) C5 convertase initiates the terminal pathway of C. The C5 molecule is homologous to C3 and C4, but lacks the internal thioester bond. Cleavage of C5 releases the small anaphylactic fragment C5a and exposes a labile binding site on C5b (Cooper and Müller-Eberhard 1970). Additionally, the activated C5b possesses an acceptor site for spontaneous binding of C6 followed by C7 causing a conformational change in the complex. The resulting C5b67 complex is released from the C5 convertase, exposing a hydrophobic region in C7 able to penetrate the target cell membrane (Preissner, Podack and Müller-Eberhard 1985). C8 is able to bind the C5b67 complex and inserts into the lipid bilayer, numerous hydrophobic C9 molecules may then polymerise into the complex (Podack and Tschopp 1982). Up to 14 C9 molecules insert into the membrane forming a ring structure resulting in a lytic pore through the membrane, abbreviated as C5b-9, or the MAC. Immunochemical studies have demonstrated that MAC deposited on lysed cell surfaces comprise on average 3 or 4 C9 molecules (Stewart et al. 1984). MAC integrated into a membrane results in a channel of approximately 100Å diameter which compromises the homeostatic capabilities of the cell and may lead to cell death (Podack and Tschopp 1982). The mechanism of pore insertion is thought to be similar to that of perforin insertion by CD8+ T-lymphocytes (Young et al. 1986).

1.2 Biological effects of C other than lysis

Whilst C activation may lead to formation of the MAC and cytolysis of target cells, a number of other biologically important effects also result from C activation. In many cases of C activation, lysis does not occur. However, in

these cases, C remains important to mediate powerful effects independent of continuation of the lytic pathway. Such consequences include the release of anaphylatoxins and deposition of opsonins each of which contribute to enhanced immune responses. A summary of the various responses attributed to C activation is shown in Figure 1.3.

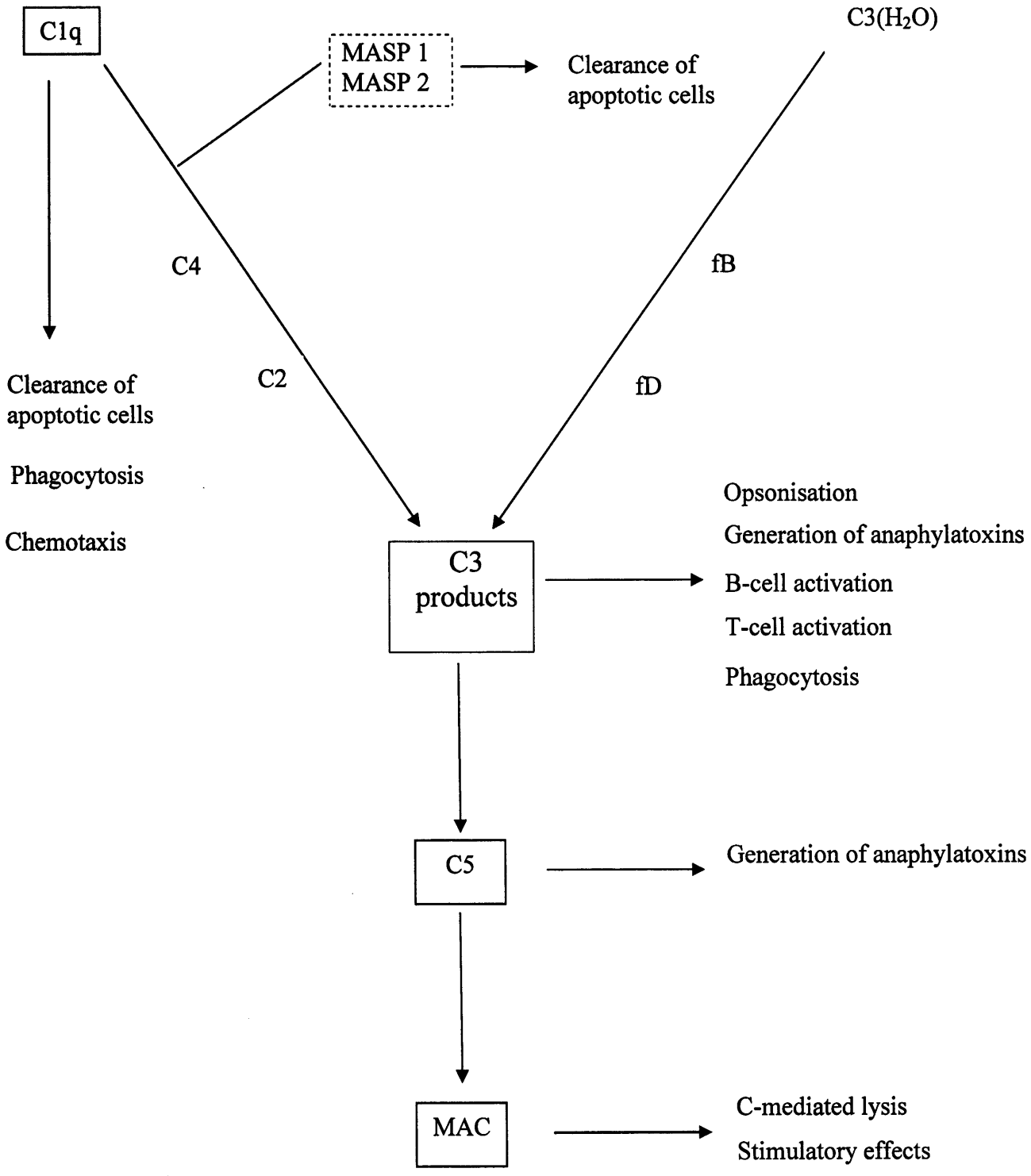


Figure 1.3: The diverse functions of C activation
The C system has numerous wide-ranging and import effects attributed to different C components. The best known effects caused by different C stages are summarised.

1.2.1 Anaphylatoxins

The smaller cleavage products of C activation do not contribute to direct lysis of a target cell. They can be, however, potent anaphylatoxins (ATs) with roles in recruitment and activation of numerous inflammatory cells around the site of C activation (e.g. C3a, C4a, C5a). The ATs are short-lived to avoid excessive inflammatory responses damaging host tissue and are rapidly inactivated by the enzyme carboxypeptidase-N. This specific plasma inhibitor cleaves an arginine residue from the C-terminal end of C3a, C4a or C5a to yield a 'desArg' form of the AT with reduced function. ATs can interact with G-protein coupled receptors (C3aR, C5aR) on various cell types to induce chemotaxis and mediate effector mechanisms of recruited cells.

C3a is released upon cleavage of C3, the central step in the C system and as such is found at higher concentrations than other cleavage products of C activation. This 9kDa fragment has been shown to mediate a number of pro-inflammatory responses (Lambris 1988). Binding of C3a to its receptor C3aR has been demonstrated to induce vascular permeabilisation and stimulate contraction of smooth muscle cells (Ember et al. 1992). Respiratory burst and subsequent production of reactive oxygen species is another major contributor to the inflammatory response and is triggered by C3a in macrophages (Murakami, Imamichi and Nagasawa 1993), and neutrophils (Elsner et al. 1994). Mast cells and basophils can also be recruited (Kretzschmar et al. 1993), and subsequently activated to release vasoactive amines such as histamine (el-Lati, Dahinden and Church 1994). Mouse cells transfected with human C3aR were shown to migrate in response to C3a but show no migratory activity in response to $C3a_{desArg}$ (Zwirner et al. 1998). However, $C3_{desArg}$ has been shown to mediate comparable effects on cytokine production by monocytes expressing the C3aR (Martin et al. 1997). This suggests that $C3_{desArg}$ may induce cellular responses through receptor independent mechanisms, however, no *in vivo* evidence is available to support this. Whilst the majority of C3a mediated effects are pro-inflammatory, a role for C3a interaction with C3aR in control of inflammatory responses has been shown via suppression of the pro-inflammatory cytokines TNFα and IL-1

(Takabayashi et al. 1996). Additionally, an anti-inflammatory role for C3a independent of C3aR signalling has been shown, resulting in secretion of pituitary hormones such as growth hormone (Francis et al. 2003).

Cleavage of C5 yields a C5a product (11kDa) and, like C3a, induces pro-inflammatory effects. Both C3a and C5a have been shown to trigger vasodilation (Schumacher et al. 1991), and induce contraction of smooth muscle cells (Ember et al. 1992). Two receptors have been identified to bind with high affinity to C5a. The vast majority of effects are mediated through binding to C5aR, a G-protein coupled receptor. The role of the alternative receptor, C5L2, remains unclear, with no signalling effects initially associated with this protein (Okinaga et al. 2003). As such, C5L2 is often referred to as the 'decoy' receptor for C5a. However, an *in vitro* study found a strong anti-inflammatory role for C5L2 and further work is needed to elucidate the *in vivo* relevance of this receptor (Gao et al. 2005). In this study, a clear role for C5L2 up-regulation in response to inflammation was shown, and a role in balancing IL-6 secretion was observed. In contrast to $C3a_{desArg}$, the $C5a_{desArg}$ fragment remains active and functions akin to C5a via binding to 5aR. A wealth of evidence exists supporting an important role for C5a in infiltration of inflammatory cells through chemotaxis (Gerard and Gerard 1994), and as such C5a is heavily implicated in allergic diseases and inflammatory disorders e.g. asthma. Degranulation of mast cells can be triggered by C5a release, leading to release of histamine, matrix metalloproteinases and inflammatory cytokines (el-Lati et al. 1994, Takafuji et al. 2003). Other cells expressing the C5aR and shown to be subject to chemoattractant effects include neutrophils (Ehrengruber, Geiser and Deranleau 1994), macrophages (Aksamit, Falk and Leonard 1981), activated B-lymphocytes (Ottonello et al. 1999) and T-lymphocytes (Nataf et al. 1999). Native C5a is more resistant than C3a to carboxypeptidase-N cleavage, and also retains biological activity in its desArg form making it the principal C derived AT *in vivo* (Bürgi, Brunner and Dahinden 1994).

C4a (Clark and Klebanoff 1978) and Ba (Hamuro, Hadding and Bitter-Suermann 1978) possess only weak anaphylotoxic effects and are likely to be of little significance *in vivo*.

Complement activation leading to enzymatic cleavage of C3 and C5 therefore contributes to inflammation via release of C3a and C5a. This may be important in the tumour microenvironment where inflammatory mediators can contribute to enhanced tumour growth e.g. TNFα and IL-6 (Lin, 2007).

1.2.2. MAC deposition

The MAC is primarily responsible for disruption of target cell membranes leading to loss of homeostasis and cell death. However, C activation does not always result in lysis of a target cell and MAC deposition can be tolerated by cells which continue to function normally. This 'sub-lytic' attack occurs when MAC is deposited in insufficient quantities to cause lysis. In these cases, activation may be low level or cells may be protected from C e.g. through expression of inhibitory molecules such as CD55 or CD59 (Kim and Song, 2006). Additionally, nucleated cells have been shown to possess intrinsic membrane repair mechanisms capable of preventing MAC deposition from resulting in lysis (Ohanian and Schlager 1981). Nucleated cells were also shown to release membrane vesicles in response to C attack (Richardson and Luzio 1980) suggesting these cells to be capable of 'shedding' the C5b-9 complex from the surface via exocytosis, a theory that was later proved using monoclonal antibodies to visualise vesiculation (Campbell and Morgan 1985). Importantly, sub-lytic attack has also been shown to confer enhanced protection to subsequent C attack (Reiter, Ciobotariu and Fishelson 1992), trigger protein synthesis and phosphorylation (Reiter et al. 1995) and stimulate cell proliferation (Niculescu, Badea and Rus 1999). Such effects are particularly significant when considering the effects of C on tumour cells and are discussed in depth in subsequent sections.

1.2.3 Opsonisation

C can also cause destruction of the target cell via another, distinct process in response to the deposition of 'opsonic' C fragments. These are C3b, C4b and associated inactivation products: iC3b, C3d and C3dg. Deposition of C3b on a cell surface is an essential step common to all 3 activation pathways but this molecule (or associated fragments) can also be recognised, and elicit a response in various cells expressing receptors specific for the opsonins (e.g. CR1, CR3). In this way opsonisation 'labels' the cell and allows enhanced adherence of many immune cell types which possess a C3b receptor (Lambris and Müller-Eberhard 1986, Fearon 1983). These receptors are found on immune effector cells and lead to enhanced phagocytosis i.e. engulfment and digestion of opsonised targets. Opsonisation provides a distinct pathway for clearance of cells independent of lysis through MAC deposition. A similar opsonic effect is mediated by C4b fixation although this effect is significantly weaker (Clark and Klebanoff 1978).

1.3. Complement Receptors

As previously described, opsonisation of surfaces by C activation products can result in adherence of various immune cell types. A number of cell surface proteins have been identified which bind to activation fragments of the C system. Four such receptors are termed 'Complement Receptor' (CR) and are numbered CR1 to CR4:

1.3.1. CR1

CR1 (CD35) is a large transmembrane protein with strong binding affinity for C3b and C4b as well as a weak interaction with iC3b. Binding of CR1 to these ligands mediates a range of functions. CR1 can act as an extrinsic co-factor to factor I facilitating cleavage of C3b to iC3b and further inactivation by cleavage to C3c and C3dg fragments (Fearon 1979). This protein is principally expressed on erythrocytes but may also be found on other cells of myeloid lineage e.g. B-cells, T-cells and neutrophils (Wilson, Tedder and Fearon 1983,

Fischer et al. 1986). A crucial mechanism for removal of immune complexes involves interaction between CR1 and C3b whereby erythrocytes 'collect' opsonised immune complexes from tissues and transport them via the bloodstream to phagocytic cells for degradation in the spleen (Cornacoff et al. 1984). CRs expressed by phagocytic cells detects opsonins on cell surfaces, and targets the cell for endocytosis (Fearon, Kaneko and Thomson 1981).

1.3.2. CR2

CR2 (CD21) was originally shown to be the receptor for the Epstein-Barr Virus (Fingeroth et al. 1984), and has been targeted to block EBV binding to lymphocytes (Nemerow, McNaughton and Cooper 1985). Human CR1 and CR2 are products of separate genes while murine CR1 and CR2 are products of a single, alternatively spliced gene. The CR2 protein is shorter and does not possess the C3b/C4b-binding site therefore cannot act as a co-factor for fI mediated cleavage. Instead, the CR2 receptor binds C3dg and C3d strongly and has only weak affinity for iC3b (Kalli and Fearon 1994). CR2 was initially isolated from Raji cells (Barel, Charriaut and Frade 1981) and is found predominantly on B-cells and follicular dendritic cells (Reynes et al. 1985).

1.3.3. CR3

CR3 (CD18/11b) is part of the integrin family of adhesion molecules but also has a distinct role in mediating phagocytosis of opsonised particles (Hogg 1992). Expressed predominantly on macrophages, CR3 binds to iC3b resulting in phagocytosis of the labelled cell.

1.3.4. CR4

CR4 (CD18/11c) also functions as an adhesion molecule and was isolated from neutrophils as a novel CR type protein capable of binding iC3b, C3dg and C3d (Vik and Fearon 1985). The primary ligand for CR4 is the C3dg molecule through a distinct binding mechanism to that of CR2. CR4 is found predominantly on neutrophils, platelets, macrophages, monocytes and erythrocytes (Myones et al. 1988). Interaction with opsonic fragments allows enhanced localisation of coated immune complexes and particles by cells of the myeloid and lymphoid lineage.

1.4. The central component C3: links to adaptive immunity

Native C3, a 185 kDa protein, is present in high concentration in the serum (1-2mg/ml) and comprises two polypeptide chains linked by a disulfide bond (Tack and Prahl 1976). C3 is central to the entire C system and common to all three activation pathways. Unsurprisingly, recurrent bacterial infection is associated with C3 deficiency through inability to cause CDC (Walport and Lachmann 1984). Outside of C activation, C3 has key roles in phagocytosis, inflammatory responses and forms important links between innate and adaptive immunity. Effects of the anaphylotoxic fragment C3a, as well as opsonic effects of C3 cleavage products have previously been described in this Chapter. Targeted disruption of the murine C3 gene generated mice lacking the majority of the functions elicited by C and has allowed better understanding of the diverse roles of C3 over the past decade (Wessels et al. 1995, Pekna et al. 1998). An overview of the diverse roles of C activation, including the contribution of C3 activation is shown in Figure 1.3.

1.4.1 C3 and the B cell response

The observation that a lack of C3 caused sub-optimal antibody responses first suggested a link between C and humoral immunity. An *in vivo* study in mice showed that depletion of C3 caused impairment of T-cell dependent antibody production (Pepys 1974). A role for the CR2 protein was initially demonstrated in enhancement of B-cell proliferation following ligation with a polyclonal antibody. This effect was only observed in the presence of T-cell factors (Frade et al. 1985). Later, an antibody targeted against murine CR2 was shown to suppress 99% of primary B-cell antibody responses *in vivo* through blocking of the binding site for opsonic fragments iC3b and C3dg (Heyman, Wiersma and Kinoshita 1990). A synergistic mechanism for enhanced B-cell responses was first described following the observation of increased Ca^{2+} influx and thymidine incorporation following cross-linkage of CR2 and membrane-bound IgM molecules on B-cells (Carter et al. 1988). The membrane localised CD19 protein was also shown to co-precipitate with CR2 and another protein CD81, with this complex shown to contribute to enhanced signal transduction (Matsumoto et al. 1991). Co-ligation of this complex with the B-cell receptor (BCR) was shown to significantly decrease the threshold for stimulation (Carter and Fearon 1992). The CR1 protein also contributes to this synergistic mechanism via co-factor activity for fI mediated cleavage thus facilitating conversion of deposited C3b to C3dg fragments and providing the ligand for CR2 attachment. This mechanism is depicted in Figure 1.4.

Therefore when antigens bind to B-cells through the BCR, opsonic C fragments (C3dg / C3d) on antigen surfaces can also bind CR2 expressed on B-cells. CR2 forms part of the CR2/CD19/CD81 complex and contributes to enhanced B-cell signalling and activation resulting in a more efficient antibody response. Further work by Dempsey et al (1996) demonstrated a role for CR2 binding to antigen opsonised with C3d in lowering the threshold for B-cell activation. In this study, immunisation of mice using C3d fusion proteins lowered the amount of antigen required to elicit a secondary response by 10,000 fold through co-ligation with CR2 and was shown to be dependent on amount of C3d attached to antigen.

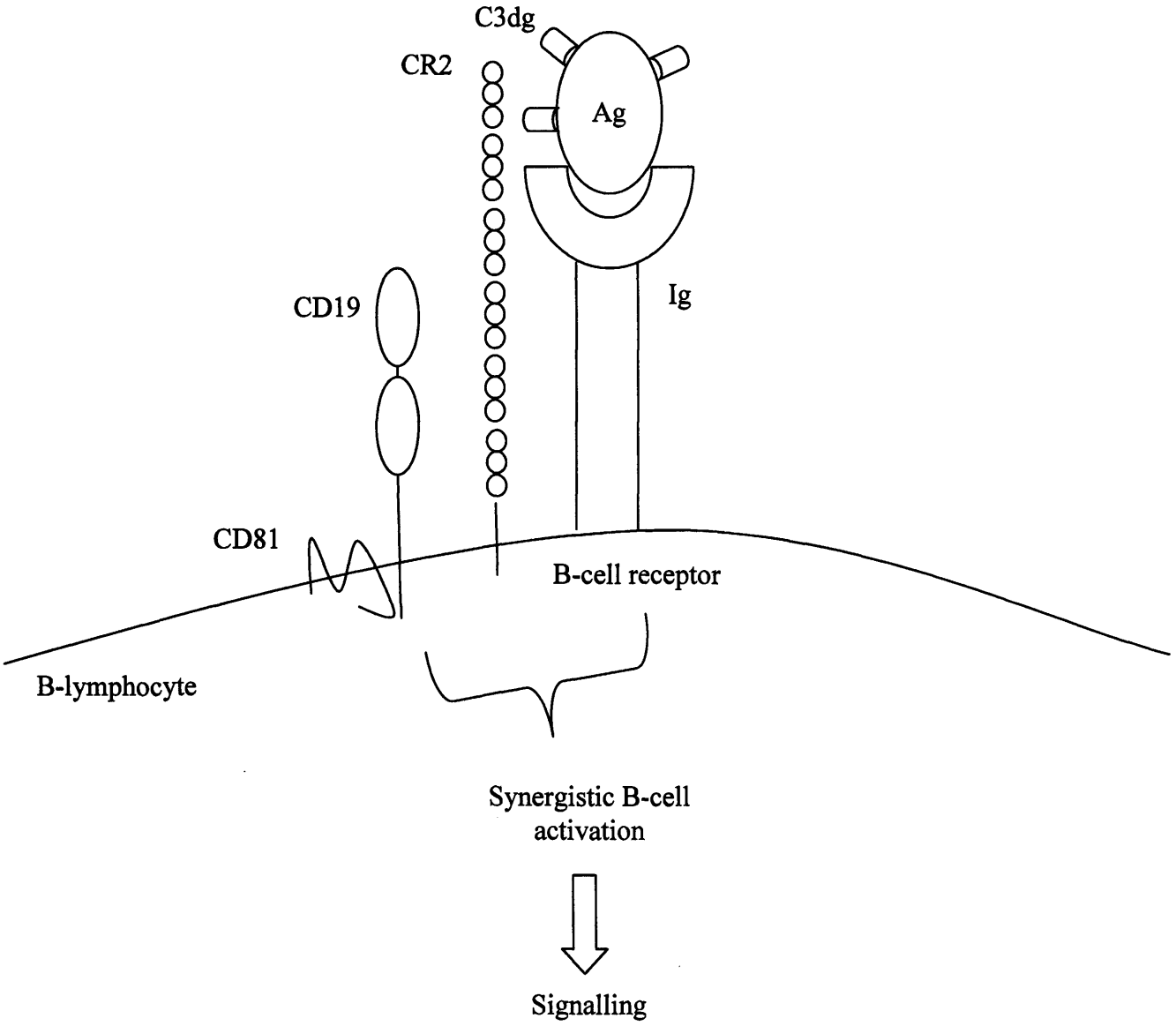


Figure 1.4: Antigenic opsonisation contributes to B-cell responses
Antigen opsonised with the C3dg fragment can bind with CR2 in a complex with CD19 and CD81. Co-stimulation of this complex with the B-cell receptor results in a synergistic response and contributes to optimal humoral immune responses.

1.4.2. C3, dendritic cells and the T-cell response

A role for C as an accessory molecule in the adaptive immune system has been the subject of much recent research with evidence available to demonstrate a modulatory role for C3 in T-cell activation and migration. Dendritic cells (DCs) are heterogeneous bone marrow derived cells crucial to the adaptive immune response as antigen presenting cells. Mature DCs are responsible for recognition and phagocytosis of pathogens, whereby antigenic fragments of degraded particles are presented on their surfaces. Subsequent activation of naïve T-lymphocytes leads to differentiation and proliferation of T-cells (both $CD4^+$ and $CD8^+$) and B-cells (Behrens et al. 2004). C has long been thought to play a role in T-cell responses and antigen-bound C3b was initially shown to enhance DC function and human T-cell activation (Arvieux, Yssel and Colomb 1988). In mice, CR1 and CR2 are expressed on activated T-cells and bind directly to C3 fragments on DCs enhancing proliferation of antigen-specific T lymphocytes (Kerekes et al. 1998). These receptors are not expressed on naïve T-cells, further suggesting a role in activation (Kinoshita et al. 1988). The presence of iC3b on apoptotic cells leads to enhanced engulfment by DCs (Verbovetski et al. 2002), with further studies on this mechanism showing important roles for CR3 and CR4 binding to iC3b (Morelli et al. 2003). Mouse DCs have been shown to secrete C3 both *in vivo* and *in vitro* with DCs derived from $C3^{-/-}$ mice shown to down-regulate MHC molecules and elicit reduced T-cell responses to stimulation (Peng et al. 2006).

A role for C3 in murine $CD4^+$ and $CD8^+$ T-cell responses to viral infection has also been demonstrated, with deficiency of C3 associated with delayed clearance of influenza virus in mice, along with increased viral titres in the lung (Kopf et al. 2002). The study also noted reduced T-cell priming of virus-specific CD4+ and CD8+ effector T cells. Importantly, these effects were not seen in CR1/CR2 deficient mice suggesting this effect was not mediated through the enhanced B-cell response described above. Instead, a C3-mediated effect independent of CR1 and CR2 was shown. An alternative receptor for this interaction has yet to be proven but may be linked to C3a and

C5a release in WT but not $C3^{-/-}$ mice. T-cell recruitment, effector function and priming were found to be sub-optimal in $C3^{-/-}$ hosts implicating C in enhancement of anti-viral adaptive immune responses.

1.5 C1q: A molecule with numerous functions

C1q, a sub-unit of the initiation molecule of the classical pathway also possesses important and wide-ranging functions unrelated to CDC. Deficiency in this protein has long been associated with autoimmune disease, with cases often leading to development of systemic lupus erythrematosus (SLE). Of 32 cases of human C1q deficiency assessed by Slingsby et al (1996), 30 went on to develop SLE while another presented with an SLE-like disease. A role for C1q in clearance of dying cells was initially proposed through stimulation of phagocytosis of opsonised cells by monocytes, mediated via the collagen-like portion of C1q (Bobak et al. 1987). A later finding showed impaired clearance of apoptotic cells (ACs) leading to SLE-like symptoms and autoimmunity though increased autoantibody production (Mevorach et al. 1998). A human keratinocyte model was used to show direct binding of C1q specifically to ACs in an antibody independent manner (Korb and Ahearn 1997). In this *in vitro* study, human keratinocytes were rendered apoptotic through UV irradiation or infection with virus. C1q binding directly to apoptotic keratinocytes was visualised using immunofluorescent microscopy and shown to be localised to apoptotic blebs implicating C1q in clearance of these cells in healthy individuals. One proposed mechanism for the clearance is through activation of the classical pathway on these cells leading to opsonisation and enhanced phagocytosis. This hypothesis is supported by the *in vivo* observation by a separate group that C3 activation products are deposited on apoptotic keratinocytes in human skin biopsies (Rauterberg, Jung and Rauterberg 1993).

Additionally, a role for C1q in directly labelling (i.e. not through classical pathway activation) these cells for phagocytosis was postulated through

interaction with C1q receptors. The generation of a murine C1q knock-out mouse colony via gene targeting has allowed rapid progress in the field (Botto 1998). Mice of this genotype showed increased mortality with glomerulonephritis and immune deposits common. A further investigation found high titres of autoantibodies and multiple apoptotic bodies in these animals (Botto et al. 1998). Activation of the CP by C1q binding was demonstrated in these mice following direct association between ACs and C1q (Nauta et al. 2002). Taylor et al demonstrated a hierarchical role for C1q and C4 in AC clearance, and found impaired phagocytosis *in vivo* (Taylor, 2000). C1q and C4 deficiencies caused significant and comparable delays in AC clearance indicating a key role for opsonisation via CP activation. However, the observation that phagocytic uptake was less impaired in $C4^{-/-}$ animals compared to those lacking C1q suggested that C1q was in fact more important in this model, thought to be mediated through direct binding to the C1qR receptor (Taylor et al. 2000). A direct mechanism for C1q binding and phagocytosis was elucidated as macrophage engulfment of apoptotic cells. This was demonstrated to be C independent and to be mediated by interaction with the membrane expressed C1q receptor calreticulin (cC1qR) and CD91 (Ogden et al. 2001). MBL has also been shown to have a similar effect on AC uptake through binding to calreticulin. Interestingly, C1q was shown to bind to both viable and apoptotic cells in this study, however only ACs stimulated macrophage engulfment. This difference is surprising and is thought to be due to localisation patterns of C1q on ACs compared to viable cells. Previous studies corroborate the authors' suggestion that 'clustered' (rather than diffuse) C1q binding in blebs is required for macrophage binding and subsequent phagocytosis (Korb et al. 1997, Navratil et al. 2001). An alternative receptor for C1q was proposed with antibody targeted against the molecule shown to inhibit enhanced phagocytosis mediated by C1q (Guan et al. 1994). This receptor was designated C1qRp and later demonstrated to be CD93 (Steinberger et al. 2002). However, transfection of CHO cells with C1qRp was shown not to confer enhanced C1q binding. Additionally, the same study found little evidence for expression on macrophages, and argues against a direct association mediating enhanced phagocytosis (McGreal et al. 2002). The same protein was later shown to enhance removal of ACs in an *in*

vivo murine model though this phenomenon was shown to be C1q independent and the role of this receptor remains controversial (Norsworthy et al. 2004).

Binding of C1q to CR1 has been demonstrated in humans (Klickstein et al. 1997), thought to aid clearance of immune complexes via binding to erythrocytes (Tas et al. 1999). C1q can also recruit, activate and cause degranulation of mast cells, central in allergic and inflammatory responses (Marshall and Bienenstock 1994) . These effects were shown to be mediated through interaction with a novel C1q receptor molecule, α2β1 integrin (Edelson et al. 2006). Antibody production by B-cells and modulation of platelet behaviour are other reported functions of C1q (Peerschke and Ghebrehiwet 1992).

Unlike the majority of C components, the primary site of synthesis for C1q is not the liver. Instead C1q has been shown to be produced locally by immune cell types, most notably macrophages on which C1q has been shown to have multiple effects (Müller, Hanauske-Abel and Loos 1978). Macrophage cytotoxicity can also be regulated by C1q through regulation of TNF-α synthesis and Nitric Oxide production (Jiang et al. 1996). C1q synthesis and secretion was up-regulated in response to stimulation in this study, while an antibody to C1q abrogated the effects. The receptor for C1q binding in these experiments was not identified. The recruitment of fibroblasts is a relevant role of C1q of particular note in this thesis. A chemotactic function of this protein has been demonstrated with human neutrophils and mouse mast cells shown to migrate in response to soluble C1q, mediated through the collagen tail (Ghebrehiwet et al. 1995, Leigh et al. 1998). C1q is also implicated in the migration of fibroblasts to injury sites (Oiki and Okada 1988), i.e. those of C activation and inflammation while an alternative effect of C1q on fibroblasts is the induction of apoptosis (Bordin and Whitfield 2003).

1.6 Regulation of C

The C system represents a powerful effector mechanism mediating numerous inflammatory effects, with the capability to destroy cells and contribute to disease. Inappropriate activation of C can have severe consequences for host tissue and is implicated in many autoimmune diseases such as rheumatoid arthritis. Rapid hydrolysis and inactivation of active fragments serves to limit the harmful effects of C activation to the vicinity of an activating surface. However, the nature of the cascade, and the possibility of active components diffusing from the site of activation has the potential to damage host cells in the vicinity, or 'bystander cells'. The classical pathway of C is antibody dependent and as such is activated only on cells which elicit a humoral (antibody) immune response. In addition, the 'tickover' of the alternative pathway and the great potential for amplification necessitates that additional protective mechanisms are in place to guard against autologous attack. A plethora of proteins exist, both membrane bound and fluid phase, with the primary function of protecting host cells from C-mediated damage. These proteins are collectively termed complement regulators (CReg) and are summarized in Table 1.1.

1.6.1 Serpins

The serpin family of proteins derives its name from the activity of its members as serine protease inhibitors. There are a huge number of serpins, wide-ranging in function and localisation. One such protein, known as C1inh, is able to prevent CP activation.

1.6.1.1 C1inh

The initiation step of the classical pathway can be inhibited by C1 inhibitor (C1inh). This enzyme inhibitor is a single chain polypeptide with variable glycosylation and like C components, is primarily synthesised in the liver (Johnson et al. 1971). However multiple other cell types have been shown to

CReg	Location	Ligand	Function
C1inh	Plasma	C1	Dissociates activated C1 complex
CD55 (DAF)	Membrane	C3bBb, C4b2a	Accelerates decay of convertases
CD46 (MCP)	Membrane	C3b, C4b	Co-factor for fI.
CR1 (CD35)	Membrane	C3b, C4b, iC3b (weak)	Cofactor for fI, accelerates decay of convertases
C4bp	Plasma	C4b, C4b2a	Cofactor for fI, accelerates CP convertase decay
Factor H	Plasma	C3b, C3bBb	Co-factor for fI, accelerates AP convertase decay
Crry	Membrane	C3b. iC3b, C4b, C4b2a, C3bBb	Cofactor for fI, accelerates convertase decay
Factor I	Plasma	C3b, iC3b, C4b	Inactivates C3b and C4b fragments
CD59	Membrane	C5b-8	Prevents C9 polymerisation by binding to C5b-8
Clusterin	Plasma	C5b-7	Prevents C8 binding to C5b-7
S-Protein	Plasma	C5b-7	Prevents C8 binding to C5b-7

Table 1.1: Summary of complement regulators
Activity and function of the complement regulatory proteins described in this Chapter.

secrete local C1inh in response to cytokine stimuli, including fibroblasts (Katz and Strunk 1989), and monocytes (Lotz and Zuraw 1987). The mode of action for C1inh is through binding to the proteases C1r and C1s causing dissociation of these molecules from C1q. This removes the enzymatic activity of C1 and prevents CP activation (Bos, 2002). C1 and C1inh proteins are both found in the fluid phase and as such, the inhibitor in circulating plasma has been shown to 'chaperone' C1 and prevent unnecessary activation (Tenner, 1986).

1.6.2 Regulators of Complement Activation

The genes encoding an important family of proteins are found in the 'Regulators of Complement Activation' (RCA) gene cluster localized on chromosome 1. Members of this family comprise varying numbers of consecutive domains known as Short Consensus Repeats (SCRs) (Hourcade, Holers and Atkinson 1989). These repeating units contain approximately 60 amino acids each and are highly conserved. Integral to the SCR structure are 4 conserved cysteine residues, these form two disulphide bridges and hold the SCR in a compact, globular conformation. The number of SCRs varies between proteins but it is these common repeating units which are responsible for interaction with activation products of C3 and C4 and thus the regulatory effects of these proteins. Early work in this field concentrated on RCA family members in humans and found the genes encoding these proteins to be closely linked and localized within an 800kb segment on chromosome 1q21 (Lublin et al. 1987, Rodriguez de Cordoba et al. 1985). The various proteins appear to be the result of gene duplication, all derived from the same ancestral gene. Investigations of RCA proteins in mice found significant sequence homology to corresponding human proteins including conservation of the SCR structures. The genes for these proteins were found located on mouse chromosome 1 (Seldin et al. 1988). However, a number of differences were also identified including designation of a new, murine specific CReg (Crry, described in section 1.6.2.6).

Human CReg which belong to this family include: complement receptor 1 (CR1; CD35), membrane co-factor protein (MCP; CD46), decay accelerating factor (DAF; CD55), C4b binding protein (C4bp) and factor H (fH).

1.6.2.1 Decay accelerator factor (DAF, CD55)

CD55 (DAF) was first described as a membrane-bound protein of human erythrocytes comprising 4 SCR domains and a molecular weight of ~70kDa (Hoffmann 1969). CD55 was purified and shown to integrate into other erythrocyte membranes and protect from C, suggesting a hydrophobic portion (Medof, Kinoshita and Nussenzweig 1984) later identified as a glycophosphatidylinositol (GPI)-anchor (Davitz, Low and Nussenzweig 1986). CD55 had previously been shown to bind the human C3 and C5 convertases of both classical and alternative pathways and accelerate their decay (Medof et al. 1984) thus regulating all three pathways of C activation, preventing further C3 deposition and initiation of the terminal pathway. Recombinant CD55 proteins deficient in individual SCRs have been used to locate areas of activity with the SCR2 and SCR3 domains shown to inhibit the classical C3 convertase and the SCR4 domain also necessary for alternative pathway inhibition (Brodbeck et al. 2000).

A functional mouse homologue for human CD55 was later identified as a ~65kDa glycoprotein (Kameyoshi, Matsushita and Okada 1989). The CD55 gene encodes a GPI-anchored form of the protein and is designated Daf-1. This protein has been found widely distributed on most tissues (Song et al. 1996). A duplicate gene is found only in mice and encodes a transmembrane form of CD55 with 78% homology to Daf-1 (Spicer, Seldin and Gendler 1995). Expression of the Daf-2 transmembrane form is restricted to the testis and spleen only (Lin et al. 2001). Targeted deletion of Daf-1 in the mouse was not sufficient to cause spontaneous lysis of erythrocytes, despite elevated levels of C3 deposition (Sun et al. 1999), although deficiency in another RCA, Crry, did cause cells to be lysed through C (Miwa et al. 2002b). However, an important regulatory function for CD55 was shown through exacerbated

phenotype in murine models of autoimmune disease (Miwa et al. 2002a, Liu et al. 2005). CD55 expression has also been shown to be up-regulated in response to cytokine stimuli and important in the protection of vascular tissue against C deposition in an inflammatory environment (Ahmad et al. 2003).

1.6.2.2 Membrane co-factor protein (MCP, CD46)

MCP was first purified from a human T-cell line (Lublin et al. 1988), and was found ubiquitously expressed except on erythrocytes (Liszewski, Post and Atkinson 1991). MCP does not exhibit decay accelerating action on either CP or AP convertases. Instead MCP possesses co-factor activity for cleavage and inactivation of deposited C3b and C4b by the serine protease factor I (Seya and Atkinson 1989). Expression of the murine MCP protein is confined to the testis (Tsujimura et al. 1998) with the role postulated to be unrelated to C regulation and important in sperm-egg interactions (Mizuno et al. 2004).

1.6.2.3. CR1

Complement receptor 1 (CD35) has previously been described in this thesis as mediating binding to C3 activation products by various immune cell types. Various forms of this protein exist with the most common variant comprising 30 SCRs and with a molecular weight of 190kDa (Klickstein et al. 1987). CR1 regulates both the classical and alternative pathways by accelerating decay of C3 and C5 convertases. An additional function of CR1 is as a co-factor for fI-mediated cleavage of C3b and C4b to inactive forms e.g. iC3b, C3dg, iC4b, C4d (Fearon 1980). In humans, the majority of CR1 is found expressed on erythrocytes and is responsible for clearance of immune complexes from the bloodstream (previously described in section 1.3.1.)

1.6.2.4. C4 binding protein (C4bp)

C4 binding protein (C4bp) is a larger plasma protein which possesses decay accelerating activity through binding to the classical pathway C3 and C5 convertases (Gigli, 1979). The protein also acts as a co-factor for fI-mediated cleavage of both surface bound and fluid phase C4b to inactive fragments (Fujita, 1978). In this way, C4bp regulates the classical and MBL activation pathways only.

1.6.2.5. Factor H (fH)

Factor H (fH) is a monomeric fluid phase protein of ~150kDa found at relatively high levels (~0.5mg/ml) in human and murine plasma. fH inhibits the alternative pathway via binding to C3b and preventing association with fB. Additionally, fH can bind to the pre-formed convertase and accelerate it's decay (Whaley and Ruddy 1976). In a similar fashion, fH promotes decay of both the classical and alternative C5 convertases (Ollert et al. 1995). As a co-factor for fI, fH also promotes the cleavage of C3b to iC3b (Harrison and Lachmann 1980).

An important splice variant of the fH gene exists, known as fH-like protein 1 (FHL-1) also with the ability to regulate the alternative pathway. Like fH, FHL-1 possesses decay accelerating activity and acts as a co-factor for fI mediated cleavage of C3b (Fontaine et al. 1989, Zipfel and Skerka 1999).

1.6.2.6. Complement receptor related protein y (Crry, 5I2 antigen)

Crry is a potent CReg found only in rodents. Initially identified during a search for a murine CR1 molecule, the 65kDa protein, initially named p65, was shown to inhibit both the classical and alternative pathways (Wong and Fearon 1985). Murine Crry is ubiquitously expressed (Funabashi et al. 1994), and provides effective protection against unwanted C attack. The molecule comprises 5 SCR domains in addition to a transmembrane and cytoplasmic

domain (Kurtz et al. 1989), and was hypothesised to function akin to MCP and CD55 (Li et al. 1993). Further study demonstrated both a C3-convertase decay-accelerating role, and fI co-factor activity for Crry (Kim et al. 1995). Crry combines the regulatory activities of MCP and CD55 and as such is of vital importance in rodents. Blockade of the protein *in vitro* caused increased AP activation with significant cytotoxicity in mesangial and endothelial cells (Quigg et al. 1995), while systemic administration of Crry neutralising mAbs is associated with increased C3b deposition, enhanced inflammation and significant cell death (Matsuo et al. 1994, Nomura et al. 1995). While CD55 retains important roles in rodents, MCP is reduced to a minimal role and is present in the testes alone (Tsujimura et al. 1998). Mice bred to be deficient in Crry died *in utero* following activation of maternal C. This embryonic lethality could be rescued by breeding to C3-deficient mice with tissue damage mediated by C deposition at the foetomaternal interface responsible for foetal death (Xu, 2000). Further evidence of the importance of Crry is provided from observations that this molecule, but not CD55 or CD59, is crucial in protection of erythrocytes from spontaneous lysis in the circulation (Miwa et al. 2002b), while rat T-cells depend solely on Crry to protect from autologous CDC (Hanna et al. 2002).

1.6.2.7. Factor I (fI)

As previously described, several of the CRegs (CR1, MCP, C4bp, fH) interact with another protein, factor I (fI). This protein is a plasma serine protease capable of regulating both activation pathways by inactivating C3b or C4b via cleavage within the alpha chain (Davis and Harrison 1982). fI can only function in conjunction with these specific co-factors but is crucial for regulation of C, in particular the amplification capacity of the AP (Nicol and Lachmann 1973). Cleavage of C3b (bound to fH, MCP or CR1) renders an inactive C3b molecule (iC3b) and another fragment C3f (Harrison et al. 1988). Further cleavage of iC3b yields C3c and membrane bound C3dg (Ross et al. 1983). Additionally, fI (in association with C4bp, MCP or CR1) can also regulate the classical pathway via cleavage of C4b to release C4c and leave

C4d attached to the membrane (Shiraishi, 1975, Fujita, Gigli and Nussenzweig 1978)). This protein is a crucial CReg and contributes to the efficacy of the ubiquitously expressed membrane-bound CReg.

1.6.3 Terminal pathway regulation

The majority of CReg exhibit inhibitory activity on the activation pathways of C whereas just one membrane bound protein has been identified able to regulate the terminal pathway. This protein, and two fluid phase proteins inhibit the terminal pathway to prevent MAC integration into target cell membranes.

1.6.3.1 CD59 (protectin)

CD59 is a member of the lymphocyte antigen 6 (*Ly-6)* superfamily, a number of related proteins of which only one is involved in regulation of C. Ly-6 family members are cysteine-rich GPI-anchored cell surface proteins forming 5 disulphide bonds within the molecule (Kieffer et al. 1994). CD59 is a small protein (19kDa) and was first purified from human erythrocyte cells (Sugita, Nakano and Tomita 1988). The activity of CD59 was shown to differ from other CReg through its action to regulate specifically the terminal pathway (Davies et al. 1989). CD59 regulates the terminal pathway by binding with the C5b-8 complex through the alpha chain of C8 (Meri et al. 1990). Whilst one C9 molecule can then bind, the polymerisation of C9 and subsequent formation of MAC is inhibited by CD59 providing a potent regulatory mechanism against MAC-induced lysis. Non-nucleated cells are more susceptible to C lysis and have been shown to express in the region of 25000 CD59 molecules per cell (Meri et al. 1990).

Murine CD59 was identified and has also been shown to be widely expressed (Powell et al. 1997). Like CD55, a duplicate gene for CD59 is present in the mouse, shown to be 63% and 85% identical in protein and gene sequence

respectively (Qian et al. 2000). CD59a is the widely distributed GPI-anchored form and is associated with the vast majority of CD59 effects. Expression of the transmembrane form of this protein, CD59b, is restricted to the testis (Harris et al. 2003).

Targeted deletion of the CD59a gene is associated with paroxysmal nocturnal haemoglobinuria (PNH)-like disease and spontaneous haemolysis in mice (Holt et al. 2001). In PNH, a stem cell mutation results in erythrocytes unable to express GPI-anchored proteins. The erythrocytes are highly sensitive to C attack leading to haemoglobinuria and anaemia in sufferers, proven to be caused by lack of terminal pathway regulation (Motoyama et al. 1992). An induced encephalomyelitis model of autoimmune disease was used to show exacerbated phenotype in CD59a$^{-/-}$ mice compared to WT characterised by rapid demyelination, inflammation and MAC deposition (Mead et al. 2004).

1.6.3.2 Clusterin (complement lysis inhibitor, CLI)

Clusterin is a ~80kDa serum glycoprotein with diverse functions. It is synthesised locally and secreted at times of cellular stress where it is involved with clearance of apoptotic cells and acts as a chaperone or may serve to inhibit apoptosis. However, a further association with C5b-9 complexes has long been demonstrated (Murphy, 1988), and shown to inhibit haemolysis in a dose dependent manner (Murphy, 1989). Further studies cast doubt on the *in vivo* relevance of this function by the observation that concentrations required to inhibit lysis were non-physiological (Hochgrebe, 1999). However, convincing evidence for a role for clusterin in Alzheimer's disease is available (reviewed in (Holtzman 2004)), with MAC deposition known to contribute heavily to the pathology of Alzheimer's disease (Shen et al. 1998). This area has been the subject of significant renewed interest following a recent landmark study identifying clusterin as a genetic risk factor for Alzheimer's disease (Harold et al. 2009). Additionally, clusterin is thought to contribute to progression of cancers (Trougakos and Gonos 2002), and has been suggested as a potential target for immunotherapy (Gleave and Chi 2005).

1.6.3.3 S-Protein (vitronectin)

S-Protein is a 75kDa serum glycoprotein able to bind fluid phase C5b-7, a precursor complex to MAC formation. Interaction between S-protein and C5-7 prevents C8 binding and insertion into the membrane. Additionally, S-Protein can interact with membrane bound C5b-8 and prevent C9 polymerisation (Podack, 1984).

1.7 Alternative roles for CRegs

Whilst the primary function of CRegs is to inhibit C and protect host tissues from autologous attack, there are numerous other roles associated with these proteins. In particular, various human and murine CRegs have been shown to have an important function in regulation of adaptive T-cell responses, signal transduction and cell proliferation.

Three CRegs of particular interest in this thesis are CD55, CD59 and Crry.

1.7.1 CD55

CD55 is an inhibitor of the activation pathways of C through action on both the classical and alternative C3 convertases. A role for this protein has also been demonstrated in T-cell responses to antigens. This was first observed in 1988 when human T-cells, incubated with phorbol esters were stimulated to proliferate via CD55 cross-linking (Davis et al. 1988). Work involving mice deficient in Daf-1, the common GPI form of the protein, demonstrated more vigorous T-cell responses to antigen than those isolated from Daf-$1^{+/+}$ mice (Heeger et al. 2005). Additionally in this study, the proliferation of $CD4^+$ T-cells was enhanced in the Daf-$1^{-/-}$ mice suggesting CD55 to have a potent down-modulatory on $CD4^+$ T-cells. Reconstitution of CD55 expression on Daf-$1^{-/-}$ T-

cells abrogated these effects showing CD55 on the T-cells themselves to contribute to the down-modulatory phenotype. However, a further observation that Daf-1$^{-/-}$ APCs stimulated stronger responses to antigen suggests that CD55 on APCs also contributes to T-cell regulation. The T-cell effects associated with CD55 were shown to be dependent on C activation through the alternative pathway since the augmented responses by Daf-1$^{-/-}$ cells were abrogated in the absence of fD. Depletion of C5 also caused partial abrogation of the enhanced phenotype with a role for C5aR in optimal T-cell responses previously reported (Kim et al. 2004).

A parallel study confirmed stronger T-cell responses to antigen in Daf-1$^{-/-}$ mice and also showed more vigorous responses to re-stimulation (Liu et al. 2005). This effect was also shown to be dependent on C activation and deposition of C3b and was abolished in the absence of C3. A notable difference in this study compared to the previous report was the finding that effects were elicited through CD55 expression on the lymphocytes only. Data provided by Liu et al. showed that APCs from Daf-1$^{-/-}$ and Daf-1$^{+/+}$ were able to stimulate comparable T-cell responses to antigen rather than the augmented responses elicited by Daf-1$^{-/-}$ APCs noted by Heeger et al.

The role of CD55 as a potent CReg appears critical to the observed effects in these studies whereby inhibition of convertase assembly, and inactivation of C3b by CD55 is mediating the effects on T-cells. In the absence of CD55, C-activation is enhanced leading to elevated levels of MAC deposition and C5a release which are able to augment T-cell responses to antigen. Further work in CD55$^{-/-}$ mice has supported a C-dependent down-modulatory role for CD55 in CD8+ T-cell responses (Fang et al. 2007).

Originally, NK-cells were thought to not express CD55 making them unusual amongst circulating lymphocytes (Nicholson-Weller, Russian and Austen 1986). However, CD55 expression was later observed at low levels on human NK-cells and a distinct sub-population identified expressing significant levels of CD55 (Solomon, Chan and Finberg 1995). NK-cell responses have been

shown to be down-regulated by CD55 (Finberg, White and Nicholson-Weller 1992, Kusama et al. 2003).

1.7.2 CD59

CD59 is a potent CReg with ability to regulate the terminal C pathway. The molecule has long been thought to play a role in human T-cell activation with various effects observed following cross-linking of CD59 with antibody. Influx of Ca^{2+}, production of IL-2, increased proliferation and tyrosine phosphorylation have been demonstrated (Korty et al. 1991, Stefanová et al. 1991). CD59 expressed on human T cells was shown to be co-stimulatory with the T-cell receptor (TCR) leading to important downstream effects including T cell proliferation. However, the same study showed an additional role for CD59 in signalling independent of the TCR (Deckert et al. 1995). Incorporation of CD59 into CD59-negative U937 cells conferred a signalling capacity through association with tyrosine kinases (van den Berg et al. 1995). More recent studies have shown roles for CD59 in enhancing human NK-cell cytotoxicity whereby CD59 expressed on NK-cells themselves was shown to have a co-stimulatory role with the natural cytotoxicity receptor (Marcenaro et al. 2003). Additionally, transfection of U937 cells with CD59 enhanced NK cell responses following antibody cross-linking due to signalling via its GPI-anchor (Omidvar et al. 2006). These described roles are in direct contrast to CD55 which inhibits NK-cell cytotoxicity. A further role for CD59 has also been demonstrated in enhanced proliferation and cytokine secretion by neutrophils (van den Berg et al. 1995).

A murine model of CD59 deficiency was generated using targeted deletion of the mouse CD59a gene (Holt et al. 2001). Using these animals, Longhi et al (2005) demonstrated a down-modulatory role for CD59 on CD4+ (but not CD8+) lymphocytes. T-cell activity was enhanced through increased proliferation following CD3 stimulation. The effect was shown to require the presence of APCs but was independent of C activation and unaffected by the absence (C3-depleted and $C3^{-/-}$) of C3. This finding is of great significance

and suggests a role for CD59a in binding to APCs without engagement of C-derived ligands.

The role of CD59 as a negative regulator of murine T-cells is difficult to reconcile with the co-stimulatory role for this protein described in humans (Korty et al. 1991). However, a more recent study on human $CD4^+$ T-cells found enhanced responses to antigen following blockade of CD59 (Sivisankar, 2009). Although the mechanism underlying this effect is unclear, this study highlights a down-modulatory role for CD59 on $CD4^+$ lymphocytes supporting the findings in murine T-cells.

Both CD55 and CD59 have been shown to possess down-modulatory roles when expressed on murine T-cells. In both cases, deficiency of the CReg allowed more vigorous T-cell responses with APCs required to observe these effects. However, a significant difference between the molecules is revealed by the C-dependent nature of CD55 modulated T-cell immunity. No effects were seen in the absence of C activation in CD55-/-.C3-/- mice. This is in contrast to enhanced T-cell responses in CD59-/- mice, which were shown to be C-independent. This observation suggests the existence of an alternative ligand for CD59, which remains as yet un-identified.

An area of controversy regarding CD59 and cell signalling is the potential interaction with CD2, a signalling molecule found on both naïve and activated T-cells. Initial observations were that monoclonal antibodies targeted against CD59 restricted rosetting between T-cells and $CD59^+$ erythrocytes and that CD59 was directly responsible for the rosetting of human T-cells with CHO cells transfected with CD59 (Deckert et al, 1992, Deckert, Kubar and Bernard 1992). A binding site was later mapped for the CD59/CD2 interaction, overlapping with a site for binding with CD58 (unrelated), known to have a co-stimulatory role with CD2 (Hahn et al. 1992). However, Chinese Hamster Ovary (CHO) cells were later transfected with human CD59 and blocking mAbs used to rule out an interaction between CD59 and CD2 (Arulanandam et al. 1993). Further evidence against CD2 and CD59 interaction is provided by surface plasmon resonance (SPR) techniques which also identified no

CD2/CD59 association (Arulanandam et al. 1993, van der Merwe et al. 1994). Several further studies have been published with no irrefutable proof provided suggesting other molecules in a complex may be needed to facilitate the stimulation. It seems likely that interaction and co-stimulation may be dependent on other proteins involved in a complex and further work is required to elucidate the precise mechanism and functions of this interaction.

1.7.3 Crry

As described in section 1.5.2.4., Crry is an ubiquitously expressed CReg in rats and mice but is not found in humans. This protein has been shown to be the sole CReg protecting rat T-cells from autologous C attack (Hanna et al. 2002) and is also thought to have roles in modulation of T-cell responses. A co-stimulatory role for Crry has been demonstrated for the activation and proliferation of CD4+ T-cells (Fernández-Centeno et al. 2000). In these studies, *in vitro* co-stimulation of Crry along with CD3 antigen (ligand for the T-cell receptor) induced proliferation of a subset of $CD4^+$ primary splenocytes. In addition, co-stimulation specifically induced IL-4 secretion, with no effect observed on IFN-γ production suggesting a role in selective development of certain T-cell subsets. An independent study at the same time confirmed these findings using a different Crry-specific monoclonal antibody to co-stimulate CD3 on rat thymocytes (Arsenović-Ranin et al. 2000). A further role for Crry is provision of resistance to NK cell-mediated cytotoxicity when expressed by target cells (Caragine et al. 2002). In this study, transfection of adenocarcinoma cells with Crry conferred added resistance to NK-cell killing in a C-independent manner.

As previously described, deficiency of Crry is embryonically lethal in mice and $Crry^{-/-}$ mice could only be generated via breeding to $C3^{-/-}$ mice (Xu et al. 2000). However, $Crry^{-/-}$ mice have recently been generated in C sufficient mice by inhibition of the terminal pathway via a specific mAb (Ruseva et al. 2009). This knock-out colony will allow further investigation of the T-cell related effects attributed to Crry and their *in vivo* relevance. However, C3

consumption occurs spontaneously in these mice and native C3 is present at markedly reduced levels in serum. Therefore, while these mice are deficient in Crry, T-cell responses could be expected to be impaired *in vivo* through reduced C3 serum levels and sub-optimal opsonisation (section 1.3.2.2).

1.8 Tumour Immunity

Tumour cells differ from normal tissue cells due to a multitude of genetic and epigenetic alterations. These mutations can be spontaneous or induced through exposure to carcinogenic chemicals or radiation, while others arise following viral infection. The changes undergone by transforming cells mark tumour cells as distinct from normal host cells. They are characterised by a loss of regulation, rapid proliferation and in some cases, the ability to metastasise. Mutations in specific genes are common in numerous tumour types, one such example being the p53 suppressor gene. The protein encoded by this gene is a pro-apoptotic regulator, which binds to damaged DNA labelling the cell for apoptotic clearance. However, a mutation in this gene results in an inactive p53 protein and allows the tumour cell to evade apoptosis (Levine 1997). Mutation of this particular gene is highly prevalent in transformed cells and is seen in over 50% of all human cancers (Poremba, 1996).

1.8.1 Immunosurveillance of tumours

The concept of host immune system protecting against tumourigenesis has long been considered. The idea was first conceived by Ehrlich in 1909 who suggested that 'aberrant germs' arose continuously in our bodies but were recognized and eradicated by the immune system before becoming clinically apparent. The concept was elaborated in the 1950s through the work of Burnet and Thomas, who developed the 'immune surveillance' theory after showing immune control of transplanted tumours. The basis of the hypothesis was a constant role for the immune system in surveying the body for presence of transformed cells and destroying neoplastic cells at an early stage. Burnet

hypothesised that antigenic alterations allowed this recognition and consequent elimination by immune cells (Burnet 1957).

The use of chemical carcinogens to investigate developing tumours and the immune system gave rise to a wealth of information, both contradictory and supportive of the controversial immunosurveillance hypothesis. Evidence opposing the hypothesis was provided by a study which described nude mice (deficient in T and B-lymphocytes) which did not develop tumours more rapidly than wild type mice and that the immunocompromised mice showed no increased susceptibility to tumourigenesis (Stutman 1979). However these mice were later shown to have some limited T-lymphocyte activity, and also to possess NK cells, also implicated in tumour rejection (Hünig and Bevan 1980). The past decade has seen a resurgence of the immunosurveillance hypothesis with numerous studies supporting a role for the immune system in controlling tumour cell growth. A study involving Rag-$2^{-/-}$ mice, deficient in T-cells and B-cells showed that immunodeficient mice were at greater risk for spontaneous adenoma development (Shankaran et al. 2001). Using a selection of tumour cell lines, the authors found that 100% of tumours generated in WT mice grew in WT and Rag-$2^{-/-}$ recipients while <50% of those generated in Rag-$2^{-/-}$ mice grew when transplanted into WT recipients. This finding supported a previous study whereby tumours passaged in WT mice showed higher tumourigenic potential and grew faster than those derived from nude mice (Urban et al. 1982). A role for 'immunoselection' is supported by these studies whereby tumour cell populations subjected to positive selection pressures (i.e. a full immune response) are better adapted to *in vivo* growth in immunocompetent hosts than populations that have not undergone such a selection process.

The first human tumour antigen (MZ2-E) was identified almost 20 years ago (van der Bruggen et al. 1991), since then numerous antigens recognised by the immune system have been described. Such proteins may be specific to tumours, or up-regulated compared to normal tissues (reviewed in Pardoll 2003). In addition to antigenic differences, glycosylation patterns are altered in many tumours giving rise to novel carbohydrate patterns which may be

recognisable to the immune system (reviewed in (Hakomori 1996)). However, important to the immunoselection process is the preferential survival of tumour cells, which do not elicit strong anti-tumour responses. Consequently, tumour antigens are often expressed at low levels or elicit weak immune responses when expressed on clinically apparent tumours.

Studies identifying increased susceptibility to tumour development in immunocompromised mice have given further credibility to the concept of immunosurveillance (Cretney et al. 2002, Crowe, Smyth and Godfrey 2002, Street et al. 2002). Further, a refined model for the hypothesis defined as 'cancer immunoediting' has been proposed (Dunn et al. 2002). This hypothesis outlines three stages by which tumour cells are either eliminated, or undergo immune selection before gaining the ability to escape from immune attack:

1) *Elimination.* Nascent neoplastic cells are targeted and destroyed by T-cells and NK cells.

2) *Equilibrium.* Immune selection of tumour cells with increased resistance to immune effector cells. Contains but does not extinguish tumour cell population.

3) *Escape.* Surviving tumour cells develop effective mechanisms for immune evasion and proliferate uncontrollably.

Natural selection dictates that only those tumour cells best able to evade immune responses will go on to proliferate therefore giving rise to more aggressive malignancies. A recent study provided strong evidence to support the existence of these phases, and showed a role for T-cells and the adaptive immune system in maintaining tumours in the equilibrium phase (Koebel et al. 2007). Cells escaping immunosurveillance in this study were shown to exhibit enhanced proliferation and reduced immunogenicity compared to those remaining in the equilibrium phase. A multitude of factors influence the ability

of tumour cells to escape immunosurveillance. Strategies by which tumour cells avoid elimination by the immune system are termed 'immune evasion'.

1.8.2. Immune evasion and suppression

Mechanisms of immune evasion exhibited by tumours are wide-ranging and effective. The means by which transformed cells escape immunosurveillance represent a crucial area to be targeted in cancer therapy.

Suppression of the immune response is a crucial characteristic of tumours and is achieved by many different mechanisms, while few are well understood. T regulatory cells (TReg) are one potent cell-type harnessed by tumour cells to provide protection from host immune responses. Early work in the TReg field remained controversial until Sakaguchi et al (2005) showed a role for a CD25+ subset of CD4+ lymphocytes in regulating autoimmunity. This suppressive T-cell subset exists primarily to regulate T-cell activation and protect against autoimmunity (reviewed by Sakaguchi, 2004). However, a role in suppressing innate immune responses via production of cytokines has also been demonstrated with impaired recruitment and activity of neutrophils, macrophages and NK-cells observed following adoptive transfer of TRegs (Maloy et al. 2003). Cells of this type are best characterised via expression of the transcriptional regulator FoxP3 which is crucial for development of these cells (Fontenot, Gavin and Rudensky 2003). A role for TRegs has been proposed in suppression of anti-tumour immune responses in the tumour micro-environment with a previous report demonstrating a suppressive population of T-cells contributing heavily to tumour growth (Berendt and North 1980). TRegs have been found to accumulate within tumours and can be shown to correlate with reduced life expectancy in some studies (Woo, 2001, Petersen, 2006). Partial depletion of Tregs using CD25-specific depleting mAbs has been shown to aid immune clearance of many different tumour cell lines, including fibrosarcomas (Onizuka et al. 1999). These findings were corroborated by several other groups with cytotoxic ($CD8^+$) T-cells shown to be responsible for the tumour rejection (Shimizu, Yamazaki and Sakaguchi 1999, Sutmuller et al. 2001). Immunosuppressive effects of TReg were

subsequently shown to be mediated through endogenous production of the immunoregulatory cytokine TGF-β (Chen et al. 2005).

An alternative mechanism for immune regulation in tumours involves recruitment of myeloid derived suppressor cells (MDSCs). These cells provide pro-tumour signals and exert strong inhibitory effects on tumour infiltrating T-cells (Serafini, Borrello and Bronte 2006). MDSCs have been shown to accumulate in tumours and mediate down-modulation of immune responses, in particular the suppression of $CD8^+$ T-cells (Kusmartsev, 2005). In this study, MDSCs collected from tumour bearing mice were able to reduce $CD8^+$ T-cell responses with no effect mediated by MDSCs from naïve counterparts. The suppressive effect of these cells is mediated through production of reactive oxygen species (ROS) which act directly on CD8+ T-cells impairing their function (Kusmartsev, 2004)

1.9 Induction of fibrosarcomas using chemical carcinogen

Induction of tumours by chemical carcinogens has been invaluable in delineating roles for numerous immune cell types, cytokines and signalling pathways in tumour development. Injection of the chemical carcinogen 3-methylcholanthrene (3-MCA) is a widely used tumour induction model and has contributed heavily to the verification and refinement of the immunosurveillance hypothesis described in previous sections. 3-MCA is an insoluble poly-cyclic aromatic hydrocarbon (PAH) and causes severe tissue damage and inflammation at the site of injection. The precise mechanism of 3-MCA induced carcinogenesis is a complex and controversial subject. Whilst the toxification of PAHs *in vivo* is poorly understood, it has been shown that metabolic activation by cytochrome P450 enzymes is required to form 'genotoxic' metabolites capable of forming DNA adducts (Moorthy, 2002). The covalent attachment of these metabolites causes damage to DNA and induces significant cell death. In addition to causing severe tissue damage, 3-MCA products are highly mutagenic and can disrupt DNA in surrounding cells. As part of the wound healing response, fibroblast cells are recruited to the site

of injury and have been shown to surround 3-MCA crystals following injection (Qin et al. 2002). These cells encapsulate 3-MCA and are thus subjected to the mutagenic activity of the carcinogen. Consequently, injection of 3-MCA gives rise to fibrosarcoma tumour populations. The random mutagenic activity of 3-MCA induces highly heterogeneous cell populations which may ultimately escape immunosurveillance and form progressively growing tumours consisting of tightly compacted fibroblast-like cells. Extra-cellular matrix (ECM) secreted by fibroblasts is thought to provide a protective environment around the MCA crystals, shielding other cells from carcinogenesis as part of the host foreign-body reaction (Kovacs, 1991). It is thought that the immune system is able to recognise and eliminate some tumour cells and should insufficient mutagenesis occur before ECM encapsulation of MCA, mice remain tumour free though the carcinogen persists in encapsulated form (Qin, 2002).

Numerous factors influence tumour induction following 3-MCA injection. The dose of carcinogen administered is predictably important while different strains of mice also show different susceptibilities to tumour induction in this model (Blankenstein and Qin 2003). However, the reasons for differences observed between apparently similar studies are less clear. Identical doses of 3-MCA induced tumours in 80-100% C57Bl/6 mice in some studies (Takeda 2002, Van den Broek 1996), yet others reported far lower incidences: 40% by Smyth et al. 2000, and 20% by Cretney et al. 2002. Environmental factors specific to different animal housing facilities are likely to contribute to these observed differences. Additionally, levels of metabolising enzymes will play a role and links to gender and growth hormone have been described for these enzymes (Sharma et al. 1998, Pampori and Shapiro 1999). Other local events linked to interferon-γ responses such as inflammation, angiogenesis and T-cell responses are also important (Qin and Blankenstein 2000, Qin et al. 2003). Following a 400μg MCA dose, tumours develop with high incidence (>80%) in C57Bl/6 mice after a period of 90-150 days (Gareth Betts, personal communication, November 2005).

1.10 Activation of complement by tumour cells

As previously described, some tumour antigens may be recognised as foreign and stimulate the immune system, with one effect being production of antibodies raised against these antigens (Lloyd 1991, Houghton 1994). Such antibodies may have the potential to activate the classical pathway of C, possibly leading to CDC and destruction of the tumour cells. Due to the spontaneous activity of the alternative pathway, and the inherent C-resistance of nucleated cells, evidence for C-targeting of tumours is scant. However, support to this claim is provided by a number of reports demonstrating deposition of C activation products on various tumour cell types in humans. Antibodies targeted against C opsonins have been used to identify C deposition with persistent classical pathway activation (IgG, C3b, C4b deposition) shown in thyroid carcinoma (Lucas et al 1996), and breast cancer (Niculescu et al. 1992). Alternative pathway activation by human lymphoma (Okada and Baba 1974), and renal carcinoma has been demonstrated (Magyarlaki et al. 1996). Additionally, MBL has been shown to bind directly to human colorectal carcinoma (Ma et al. 1999), and glioma cells (Fujita et al. 1995). In these *in vitro* studies, MBL binding was demonstrated with both C3 and C4 fragments found deposited on tumour surfaces.

Activation of C classical pathway on tumour cells represents an appealing possibility for anti-tumour therapy through ability to specifically target cancer cells via monoclonal antibodies and leave normal host tissue unharmed. Over the past 5 years, the identification of suitable tumour antigens has advanced significantly and remains a priority in cancer immunotherapeutic research. However, difficulties in obtaining high titre and specificity antibodies has proved a major limitation *in vivo*, while the immunogenicity of both pAbs and murine mAbs has also proved problematic for over a decade. A promising advance was made via the development of chimeric mouse/human mAbs with reduced yet still significant immunogenicity and still further by 'humanisation' of Ab binding variable regions (Cobleigh et al. 1999). In most cases, Abs that can be effectively targeted to tumour surfaces have been used to direct

chemical agents or to block cell surface protein function. The use of C as an effector system to clear tumours is attractive due to the diverse adaptive and innate immune functions mediated by C activation. However for a variety of reasons discussed in this Chapter, C remains an inefficient mechanism for clearance of clinically apparent tumours. The majority of early anti-tumour mAbs possessed poor C-fixing capacity (Macor and Tedesco 2007), although promising evidence to support further research in this area was provided in the C-dependent control of ovarian carcinoma in humans (Macor et al. 2006). Further, it has been demonstrated that a 'cocktail' of anti-tumour mAbs could be used to significantly enhance C deposition and reduce growth of a human breast cancer line compared to treatment with individual mAbs both *in vitro* and *in vivo* (Spiridon et al. 2002). A growing number of both human/mouse chimeric and humanised anti-tumour antibodies are now available for clinical use (reviewed by Gelderman et al. 2004), and provide a potential mechanism for C-activation targeted to tumour cells. Whilst the primary anti-tumour effects of these antibodies are not C-mediated in the majority of cases, CDC has been shown to contribute heavily to tumour regression *in vivo* in some cases. The mouse/human chimeric anti-CD20 mAb Rituximab has been shown to promote CDC and ADCC in a mouse lymphoma model transfected with human CD20 (Di Gaetano et al. 2003), while Rituximab has achieved considerable success *in vivo* and is widely used in the treatment of non-Hodgkins lymphoma (Plosker and Figgitt 2003)

A current aim in C research involves harnessing the powerful effects of C to control cancer. Several anti-tumour antibodies have been shown to activate C *in vitro* (Sliwkowski et al, 1999, Trikha et al. 2002). However, the expression of CRegs remains a major obstacle and has been shown to limit the efficacy of anti-tumour therapeutic mAbs (Golay et al. 2001). Further progress in this area may be achieved through modification of C-fixing capacity of antibodies to optimise C activation e.g. by preferentially engineering IgG1 or IgG3 mAbs will increased C activation efficacy (Idusogie et al. 2001). An extremely attractive mechanism to circumvent this problem is through the use of bi-specific Abs which co-target a tumour antigen and CReg. In this way, a therapeutic Ab could simultaneously target a tumour cell, and partially

neutralise the ability to regulate C. The use of a chemically engineered construct, able to simultaneously bind tumour cells and neutralise CD59 allowed efficient killing of Raji cells *in vitro* (Harris et al. 1997). A further bi-specific Ab directed against renal tumour antigen G250 and the CReg CD55 was shown to cause enhanced tumour cell lysis compared with anti-G250 alone with increased C3b deposition demonstrated (Blok et al. 1998). Further demonstrations of the use of bivalent Abs incorporating an anti-tumour moiety and capacity to neutralise a CReg have been provided *in vitro* using a human and rat model of colorectal cancer (Gelderman et al. 2002b, Gelderman et al. 2004). This technique may be applicable to many tumour cell types and remains a promising mechanism in tumour immunotherapy. Bi-specific anti-CD20 with either human CD55 or CD59 has been suggested as an adjuvant to conventional Rituximab anti-lymphoma therapy with a two-fold increase in CDC demonstrated *in vitro* (Ziller et al. 2005).

1.11 Protection of tumour cells from complement

All host cells require effective mechanisms to protect them from the damaging effects of autologous C. Tumour cells may also utilise and selectively regulate these protective mechanisms to confer added resistance.

1.11.1. Membrane bound CReg

A major limitation to the efficacy of C in tumour immunosurveillance is provided by the presence of CReg expressed by tumour cells. As previously described, these proteins are secreted or expressed on host cell membranes to protect from autologous C and autoimmunity. The presence of these CReg on the tumour cell surface will afford effective protection against C mediated attack, providing a significant obstacle to the use of C in anti-tumour therapy. In addition to the levels of protective molecules expressed by normal tissue, a wealth of evidence exists to support modulation of expression patterns of these proteins on tumour cells (Reviewed by Fishelson, 2003). In the majority

of cases, CRegs are over-expressed in transformed cells conferring enhanced protection against C effector systems. A proposed explanation for this is that CDC or opsonisation may lead to clearance of poorly protected cells, and select for high CReg expressors. Alternatively, CReg expression has been shown to depend on differentiation stage *in vivo* with CD59 expression increased in well differentiated human carcinomas as compared to little or no CD59 expression in poorly differentiated cells (Koretz et al. 1993). In order to exploit C as a potential effector system in tumour immunotherapy, neutralising antibodies to CReg have been used with variable success. A wealth of evidence exists demonstrating that blockade of various CReg to effectively sensitise *in vitro* cell lines to CDC. Examples of this include neutralisation of CD59 on human neuroblastoma cells (Gasque et al. 1996), and both CD55 and CD59 on human breast carcinoma and leukaemic cells (Golay et al. 2001). Neutralisation of CD55 and CD59, but not CD46, has also been used to sensitise erythroleukaemic K562 cells to CDC (Harris et al. 1997, Jurianz et al. 2001), while blockade of CD55 or CD59 significantly enhanced C3 deposition on human cervical carcinoma cells (Gelderman et al. 2002a). An important development in this area was provided by the use of bi-specific neutralising antibodies (described in section 1.10). In this way, cells can be co-targeted with an anti-tumour effector antibody alongside a CReg neutralising antibody. This therapeutic technique inhibits CReg only on tumour cell surfaces and prevents significant damage to bystander cells. Co-targeting of tumour antigens with anti-CD55 or anti-CD59 antibodies have been shown to significantly enhance killing of both mice and human tumour cells *in vitro* (Harris et al. 1997, Blok et al. 1998). This technique was also utilised in a rat model of lung metastasis whereby tumour-specific blockade of Crry significantly decreased metastases in the lung, with enhanced C3b deposition observed (Gelderman et al. 2004). Further *in vivo* work in this area is required to assess the feasibility of modifying current mAb immnunotherapies to incorporate CReg blocking moieties and increase CDC and opsonisation. In addition to Ab blockade of CReg, anti-sense oligonucleotides can be used to sensitise tumour cells to *in vitro* CDC by decreasing CReg expression (Zell et al. 2007). However, a major obstacle to this technique is the current lack of method for specifically targeting CReg down-regulation to tumour cells.

The over-expression of CReg by tumours has been shown to correlate with poor patient prognosis in colorectal cancer patients (Durrant et al. 2003, Watson et al. 2006). Additionally, CD55 has been linked to proliferation rates with over-expression of this CReg by colorectal cancer cells shown to exhibit enhanced proliferation. In direct contrast to this observation, loss of CD55 was demonstrated on advanced stage breast carcinomas and the lack of CD55 was shown to correlate with poor prognosis (Hofman et al. 1994, Madjd et al. 2004). Despite these seemingly contradictory reports, CD55 has been implicated in discriminating tumour grade i.e. stage and suggested as a potential cancer vaccine for T-cell immunotherapy (Spendlove et al. 1999). Initially, CD55 appears a poor candidate for tumour immunotherapy due to its widespread expression in humans and important role in regulating C activation. However, a tumour associated antigen was identified in an osteosarcoma cell line by a mAb raised in mice immunised against this cell line (Embleton et al. 1981). This antigen, designated 791Tgp72, has been described as a 72kDa glycoprotein commonly found over-expressed in various tumour cell types including osteosarcomas and colorectal carcinomas (Pimm et al. 1982, Durrant et al. 1986). The mAb recognising this antigen, 791T/36, has been used to effectively and selectively image tumour cells and predict patient prognosis (Powell et al. 1987, Buckley 1989, Austin, 1989). One such mAb, named 105AD7, mimics the 791Tgp72 antigen and has been used in colorectal carcinoma patients to prolong survival by stimulating anti-tumour T-cell responses. $CD4^+$, $CD8^+$ T-cell and NK-cell responses appeared higher in those patients vaccinated with 105AD7 and delayed tumour growth (Buckley, Robins and Durrant 1995). The 791Tgp72 was later identified as showing 100% sequence identity to CD55 and specifically binds anti-CD55 antibodies (Spendlove et al. 1999). This discovery was unexpected given the ubiquitous expression of CD55 in humans and the non-toxic effects of immunising with 105AD7 which effectively mimics 791Tgp72 i.e. CD55.

The reasons underlying the unlikely identification of CD55 as a tumour antigen is due to differential antigen density and expression patterns in the tumour microenvironment compared with normal tissue. The enhanced T-cell

mediated cytotoxicity specific to tumour cells in patients immunised with 105AD7 is likely caused by the over-expression of CD55 (<100-fold) observed on tumour cells compared to normal cells (Li et al. 2001). In this way, tumour cells may express low levels of CD55 thus being more susceptible to CDC, or over-express CD55 and present a promising target for vaccination and T-cell immunotherapy.

A number of different isoforms of CD55 exist, shown to result from different glycosylation patterns in colorectal carcinomas (Nakagawa et al. 2001). This results in heterogeneous molecular weights observed for this protein and these tumour-specific isoforms also provide therapeutic targets. The SC-1 mAb is known to bind a high molecular weight (~82kDa) isoform of CD55, preferentially expressed by stomach carcinoma cells. SC-1 binding induces cell phosphorylation and apoptosis (Hensel et al. 1999). Such effects are independent of CDC and ADCC and have negligible detrimental effects on normal immune responses making this an attractive anti-tumour therapy (Vollmers et al. 1998, Beutner, 2008).

1.11.2. Soluble CReg

In addition to up-regulation of membrane-bound regulatory proteins, tumour cells may secrete soluble CReg to inhibit C in the tumour micro-environment. Fluid phase regulators have been associated with secretion by tumours including C1inh by a neuroblastoma line (Gasque et al. 1996). fH, a potent inhibitor of the alternative pathway, can be secreted by certain types of tumours including human ovarian cancer cells and this secretion and subsequent binding was shown to provide a novel mechanism aiding resistance to C and contributing to the strong resistance of glioblastoma cells to CDC (Junnikkala et al. 2000, Junnikkala et al. 2002). Expression of fH has also been put forward as a possible marker for bladder cancer (Kinders et al. 1998). Binding of fH to phosphoproteins up-regulated in certain tumour types has been shown to directly contribute to tumour cell resistance to CDC of mouse erythroleukaemic cells and human breast carcinoma lines (Fedarko et al. 2000), while down-regulation of fH by siRNA caused retarded *in vivo*

growth of human lung carcinoma cells through a C-dependent manner (Ajona et al. 2007). Factor I has been detected in the supernatant of a sarcoma line (Legoedec et al. 1995), and shown to effectively cleave deposited C3b and C4b when secreted by non-small cell lung carcinomas (Okroj et al. 2008).

Soluble forms of membrane bound CReg can also be released from cells to supplement membrane bound CRegs. The released forms may be synthesised without the GPI anchor or may be enzymatically cleaved from the surface. Increased soluble CR1 has been observed in leukaemic patients and elevated soluble MCP levels observed in several tumour types (Seya et al. 1995, Sadallah et al. 1999). Both CD55 and CD59 were observed at high levels in tumour stroma of various carcinomas (Niehans et al. 1996). Further studies found that CD59 can also be secreted by melanoma cells (Brasoveanu et al. 1997), while CD55 was found to be deposited into extra-cellular matrix at levels proportional to surface levels in osteosarcoma and colorectal carcinoma (Li et al. 2001).

1.11.3. Ecto-proteases

Other proteins have been demonstrated to influence C activation and cleave opsonic fragments on cell surfaces. This may result in activation of C through the amplification loop of the alternative pathway, or, inactivation of opsonins leading to protection from C and phagocytosis. Amongst these secreted proteins are a group known as ecto-protease enzymes with the capability to cleave C components, in most case the activation products of the central component C3. These proteases are not specific to tumours and can be secreted by various cell types (Hermann, Barel and Frade 1994). Human melanoma cells expressing one such protein, p65, have been shown to be highly resistant to CDC, with antibody blockade of p65 abrogating this resistance and sensitising cells to lysis (Panneerselvam et al. 1986, Ollert et al. 1990). A C3-cleaving cysteine proteinase was identified in another human melanoma study, termed p41. Expression of this proteinase was found to coincide with secretion of tumour derived C3 and this co-secretion to correlate

with a high metastatic potential (Jean, 1997). A further C3-cleaving proteinase (p39) was identified in mouse melanoma cells and shown to share sequence identity with procathepsin-L (Jean, 1995), also demonstrated to confer high tumourigenicity in human melanoma lines (Frade et al. 1998). The enzymes described in this study cleave native C3 with C3b and C3dg fragments detectable in supernatants. These fragments may deposit on antigenic surfaces leading to opsonisation and/or continuation of the C pathway, or may interact with various receptors to regulate cellular functions.

1.11.4. Sialic Acid

Cell surfaces expressing high levels of polyanionic molecules are protected from C activation through enhanced binding of fH to C3b. This binding competes with fB binding and enhances inactivation by fI-mediated cleavage in the presence of polyanions (Pangburn and Müller-Eberhard 1978). Therefore, high levels of sialic acid confer enhanced resistance against amplification of the alternative pathway and CDC (Meri and Pangburn 1990). Sialylation is implicated in protection of many host cell types from excessive C3b deposition and protection from autologous attack. Unsurprisingly, sialic acid is also important in tumour resistance to CDC and bladder cancer cells can be markedly sensitised to C-mediated lysis following removal of sialation by neuraminidase enzyme (Jacobsen et al. 1982). Further, three human carcinoma cell lines have been shown to express high levels of sialation correlating with resistance to CDC (Donin et al. 2003). Protection of tumour cells from C by high expression levels of sialic acid has also been observed in murine leukaemic lines (Shi et al. 1996). In each of these studies, removal or blocking of sialic acid binding was shown to sensitise tumour cells to CDC showing an important role for the non membrane-bound CReg fH in tumour protection from C and suggest a possible mechanism for targeting of tumour cells.

1.12 Sublytic effects of C

The major effector molecule of CDC is the C5b-9 MAC complex which forms a transmembrane channel and compromises homeostatic capabilities of target cells. Whilst C is able to lyse bacterial cells and erythrocytes effectively, nucleated cells possess additional mechanisms to prevent MAC-mediated lysis and are significantly more resistant to CDC (Ohanian and Schlager 1981). This was evidenced by a study on nucleated cells which required co-operative channel formation in a multi-hit manner to cause lysis (Koski et al. 1983). Demonstration of cellular responses to MAC deposition was provided by the initial observation of Ca^{2+} influx and inhibition of cyclic AMP formation in the absence of cell lysis (Campbell et al. 1981, Hallett et al. 1981). Further, U937 cells were shown to be capable of rapidly eliminating MAC molecules from their surface in a process not observed on erythrocytes (Ramm et al. 1983). The mechanism for this process was elucidated via the use of a mAb specific for C9 which showed vesiculation and removal of MACs from leukocyte cell surfaces (Campbell and Morgan 1985). This effect was shown to be temperature dependent and associated with increased metabolism. A later study found that human neutrophils rapidly eliminate all MAC molecules from cell membranes with both vesiculation and internalisation processes observed (Morgan et al. 1987).

A variety of effects have been associated with sub-lytic deposition of MAC on nucleated cells. However the mechanisms underlying these effects, and the specific functions attributed to the changes remain poorly understood. The immediate influx of Ca^{2+} is critical to mediate these effects and protect nucleated cells from CDC (Morgan and Campbell 1985). Additionally, lipid metabolism has also been shown to contribute to cell survival following MAC deposition (Ohanian and Schlager 1981, Papadimitriou et al 1991). The protein kinase C family (PKC) is known to play a role in cellular activation events following the abrogation of protection after treatment with PKC inhibitors (Kraus and Fishelson 2000). Cell signalling events associated with these enzymes were shown to involve the ERK mitogen-activated protein

kinases (Kraus, Seger and Fishelson 2001). Phosphorylation of these kinases can regulate numerous downstream effects including regulation of gene expression, apoptosis and cell proliferation (Pearson et al. 2001). In addition to this signalling cascade, activation of the Ras GTPase (Niculescu, Badea and Rus 1999), and c-Jun families has also been demonstrated (Rus et al. 1997), with roles in cell proliferation and cell cycle phase transition respectively. Sublytic MAC has been shown to trigger *de novo* protein synthesis in both normal (Benzaquen, Nicholson-Weller and Halperin 1994), and tumour cells (Reiter, Ciobotariu and Fishelson 1992). The above observations, and the presence of intrinsic cell repair mechanisms, have led to the hypothesis that sublytic MAC deposition is capable of activating cells and providing supplementary resistance to basal C-protective mechanisms.

A further observation following sublytic MAC deposition on nucleated cells is a phenomenon known as 'C-induced protection'. This *in vitro* effect was described following treatment of human leukemic lines with a sublytic C dose. Following recovery of cells, subsequent treatment of cells with a lytic dose was unable to cause lysis (Reiter et al. 1992). Thus, cells surviving initial MAC deposition were conferred an elevated level of resistance against further C-activation with the effects shown to be associated with Ca^{2+} influx and increased protein synthesis. The induced protection was shown to be independent of MAC deposition levels and was lost after 10-12 hours. In addition to enhanced protection from C, cells are also rendered resistant to lysis by other pore-formers e.g. perforin (Reiter et al. 1995). A later study showed negligible lysis of K562 cells treated with 16-fold higher C dose than that required to lyse >70% cells prior to sublytic attack (Marchbank, van den Berg and Morgan 1997). Importantly, Experiments performed in this study ruled out differential binding of C-fixing Ab and CReg expression as potential contributors to the protected phenotype. A weaker but still significant effect was found on U937 cells in the same study. An additional consequence of sublytic MAC deposition on K562 cells (and other human leukaemic lines) is the rapid production of a large C induced protein (L-CIP)(Reiter and Fishelson 1992). This protein is related to the heat shock protein family and is thought to play a role in tumour cell escape from CDC (Fishelson et al. 2001). The

mechanism for C-induced protection remains unclear. However, the consequences of C-activation failing to lyse targets may be of considerable significance *in vivo*. In particular, tumour cells which are known to activate C and deposit activation products may be subjected to the various stimulatory effects associated with MAC deposition and gain acquired resistance to C and associated immune mechanisms.

Aside from MAC deposition, C3 activation fragments are able to increase the proliferation of B-cells by association with CR2 (see section 1.4.2.1). However, direct binding of C3 products has also been implicated in promoting cellular proliferation. Lewis lung carcinoma cells have been shown to bind C3 fragments independent of CRs (di Renzo et al. 1999). The fragments were internalised and were shown to contribute to enhanced proliferation of the cells *in vitro*. The authors suggested that C3 fragments are translocated from the surface and provide a stimulatory growth signal. The effects were shown to be independent of MAC deposition and were abrogated in the presence of anti-C3 Abs. Further study by the same group found that C3 fragment internalisation was mediated by PKC activity (Longo et al. 2005), and postulated the enhanced proliferation to be mediated through previous correlations between PKC and cell growth rate in tumours (Matsuzaki et al. 2004). Proliferative effects were abrogated in the presence of PKC inhibitors while initial C3b binding to cell membranes was un-affected suggesting a role for PKC in the internalisation process rather than initial binding.

Whilst the majority of studies involving C and tumours have involved targeting basal resistance mechanisms e.g. CReg, induced effects remain an important consideration and an area requiring significant further investigation. Effects by which C activation on cellular surfaces may contribute to enhanced survival or proliferation crucial to the emerging hypothesis by which C, below the threshold of killing, may represent a pro-tumour stimulus and promote tumour progression.

1.13 Secretion of complement components by tumours

As described previously, the vast majority of C proteins are synthesised in the liver, though numerous cell types have been reported to synthesise C components locally (Morgan et al. 1997). A number of reports over the past 20 years have identified C component synthesis by transformed cells, in the majority of cases this involves the central C component C3. Unsurprisingly, hepatoma cells from humans (Falus et al. 1990), rats (Stapp et al. 2005), and mice (Lin et al. 1993), have all been shown to secrete C3. However, further reports of C3 secretion by alternative cell types may indicate a pro-tumour role for C3 secretion. The strongest evidence for this is provided by a study in which secretion of C3 was found to correlate with increased metastasis in human melanoma cells (Jean et al. 1997). Important to this study was the identification of a C3-cleaving enzyme, p41, found to be co-secreted with C3 (Section 1.11.3). Whilst expression of p41 alone may be sufficient to promote metastasis of these cells, a selective advantage to tumour cells secreting their own C3 may also contribute. Co-secretion of C3 and a p41 may represent a tumour mechanism able to harness the stimulatory effects of C3b deposition (as described in Section 1.12) whilst avoiding CDC. Human gastric cell lines (Kitano et al. 1993), colorectal carcinoma (Andoh et al. 1998) and myoblasts (Legoedec et al. 1995) have all been shown to up-regulate C3 synthesis in comparison to normal cells derived from the same tissue. Production of various pathway components have been demonstrated in astroglioma cells (Barnum et al 1992), and activation of C3, C5 and C9 genes observed following treatment of lung carcinomas treated with photodynamic therapy (Stott et al. 2007). A clear role for tumour derived C components is yet to be defined and may be simply be a result of loss of gene control common in tumour cells. However, tumour-derived C may be significant to the emerging hypothesis that tumour cells can 'harness' the C-system to provide stimulatory and proliferative effects whilst avoiding CDC. Further investigation into the contribution of C-derived C components in tumour progression is needed.

1.14. Aims of this thesis

The relationship between the C system and tumour immunity is poorly understood. Whilst C components can undoubtedly be activated and deposited on tumour cell surfaces, this does not lead to clearance either by CDC or phagocytosis following opsonisation with C products. The conventional role of C, and the described reports of deposited C activation fragments on tumour cell surfaces have previously suggested a role for C in immunosurveillance and in anti-tumour responses. However, an alternative hypothesis is now emerging whereby C activation on tumour cell surfaces may lead to enhanced C-resistance, proliferation and metastasis. Deposition of C3b and MAC on nucleated cells has been shown to mediate various stimulatory effects including cell activation. In this way, C may actually contribute to tumour progression.

Mice deficient in specific C components provide an invaluable tool allowing detailed investigation of the respective roles played by individual proteins in tumour rejection or progression. The chemical carcinogen, 3-MCA, can be used to induce fibrosarcomas in wild type and C knock-out mice unable to elicit specific C-mediated effects. Differences in tumour survival and growth are expected dependent on whether the C component is contributing to tumour immunosurveillance or progression. From tumours induced in WT and C-deficient mice, novel tumour lines can be generated *ex vivo*, and characterised *in vitro*. The re-inoculation of cells sufficient or deficient in synthesis of C components or regulators into WT and C-deficient hosts provides a powerful model by which the role of cell-derived and systemic protein can be investigated. A protein of particular significance is the central C component C3. Table 1.2 outlines a typical experiment designed to address this issue:

Tumour Line	Recipient Animal	Complement Source
C3-	$C3^{-/-}$	No C3
C3+	$C3^{-/-}$	Local (tumour C3 only)
C3-	$C3^{+/+}$	Systemic C3
C3 +	$C3^{+/+}$	Local & Systemic C3

Table 1.2: Control over host/tumour derived C3 secretion.
Mice sufficient and deficient in C3 are injected with fibrosarcoma cells with and without the ability to synthesise C3. In this way, the effects of systemic and tumour-derived C3 on fibrosarcoma incidence or growth can be assessed.

Aims:

In order to investigate the relationship between C and tumour growth, the specific aims of this thesis were:

1) To optimise a method for, and to investigate the effect of a deficiency in a C component or regulator on in vivo growth of a pre-characterised WT fibrosarcoma line. This work is described in Chapters 3 and 4.

2) To investigate the effect of a deficiency in a C component or regulator on tumour induction by the chemical carcinogen 3-MCA. Differences in tumour survival, progression and phenotype are described in Chapter 5.

3) To excise tumours induced in WT and C-deficient mice, and to generate and characterise novel fibrosarcoma lines *in vitro* from these tumours. To assess *in vivo* growth of these lines and to examine the effect of C produced by the tumours themselves, or systemic C, on tumour growth. This work is described in Chapter 6 and is illustrated in Table 1.2 above.

2. Materials and Methods

Coomassie Brilliant Blue Stain: 0.2% Coomassie Blue R250 in 45% Methanol, 10% Acetic acid in dH_2O.
Destain: 20% Methanol, 7% CH_3COOH in dH_2O.

Gel Drying Buffer: 4% Glycerol, 20% Methanol in dH_2O

Flow Cytometry Buffer: PBS, 1% BSA (Sigma), 1% NaN_3, pH 7.4.

Phosphate Buffered Saline (PBS): 8.1mM Na_2HPO_4, 1.5M KH_2PO_4, 137nM NaCl, pH 7.4.

PBS-BSA: PBS, 1% w/v Bovine Serum Albumin (BSA).

PBS-Milk: PBS, 5% w/v dry skimmed milk powder.

PBS-Tween: PBS, 0.1% Tween 20 (Acros Organics).

SDS-PAGE Stacking Gel Buffer: 0.5M Tris, 0.4% (w/v) SDS, pH6.8.
SDS-PAGE Resolving Gel Buffer: 1.5M Tris, 0.4% (w/v) SDS, pH8.8.

SDS-PAGE Running Buffer: 25mM Tris, 191mM glycine, 1% (w/v) SDS.

SDS-PAGE Loading Buffer (reduced): 0.1M Tris (pH6.8), 10% (v/v) Glycerol, 2% (w/v) SDS, 0.01% Bromophenol Blue, 2% (v/v) β-mercaptoethanol (β-ME). β-ME omitted for non-reduced samples.

Transfer Buffer: 25mM tris, 191mM glycine, 20% methanol.

ELISA Coating Buffer: 0.2M Na_2CO_3, pH9.6.

ELISA Development Solution: 4x OPD tablets (Dako) added to 12ml dH_2O, 5µL 33% H_2O_2.

Complement Fixation Diluent (CFD): 2.8mM Barbituric acid, 145.5mM NaCl, 0.8mM $MgCl_2$, 0.8mM $CaCl_2$, 0.9mM Sodium Barbital, pH 7.2.

Erythrocyte Lysis Buffer: 8.3g NH_4Cl, 1g $KHCO_3$, 37mg potassium-EDTA, pH 7.4

Table 2.1: Buffer compositions for methods detailed in Chapter 2

2.1. Mice

Mice were bred in Specific Pathogen Free (SPF) conditions and maintained in filter-top cages in Biomedical Services Unit, Cardiff University, Heath Park. $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice were previously back-crossed onto C57BL/6 background at least 10 times (Morgan et al. 2006). Knock-out mice were controlled with age-matched C57BL/6 WT mice. Adult mice between 6-10 weeks of age were used for all experiments. All experiments were performed under license and in compliance with Home Office regulations.

2.2. 3-Methylcholanthrene tumour induction

Four hundred µg 3-MCA (Sigma-Aldrich) was injected in a 100µL dose sub-cutaneously on the mouse hind leg to induce fibrosarcoma tumours. 3-MCA was dissolved in olive oil immediately prior to use. Mice were monitored weekly for tumour development using callipers (Jencons) to measure tumour width and length. Tumour volumes were estimated according to the formula: $(\pi \times \text{length} \times \text{width}^2) / 6$ (Jones et al, 2002). Mice were sacrificed when tumour diameter reached 1cm.

2.3. Cell culture

Fibrosarcoma cell lines were grown in RPMI-1640 (Gibco) supplemented with 10% (v/v) heat inactivated foetal calf serum, 50U penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1% non-essential amino acids (Invitrogen) unless otherwise specified. Cells were maintained with 30ml media in T175 tissue culture flasks (Nunc) unless otherwise stated and incubated at 37°C, 5% CO_2. When confluent, flasks were washed twice with sterile 0.9% NaCl solution (Fresenius Kabi Ltd) and incubated (10 minutes, room temperature) with 0.5ml Versene (1:1000 EDTA, Invitrogen), per flask to lift adherent cells. Cells were re-suspended in 10ml fresh media and centrifuged (800g, 3 minutes) to pellet. Resulting pellet was re-suspended in 5ml media, mixed briefly and 1ml removed to seed a new T175 flask.

Antibody	Specificity	Isotype	Application	Dilution	Source
Mel4	Rat anti-mouse CD59 mAb	IgG1	WB, FC, IHC	10µg/ml	In house
MD1	Rat anti-mouse CD55 mAb	IgG2c	WB, FC, IHC	10µg/ml	In house
5D5	Rat anti-mouse Crry mAb	IgG1	WB, FC, IHC	10µg/ml	M.V.Holers
Apa5	Rat anti-mouse CD140a mAb	IgG2a	WB, FC	10µg/ml	e-bioiscience
11H9	Rat anti-mouse C3, C3b, iC3b, C3dg mAb	IgG2a	FC, IHC, ELISA	10µg/ml 5µg/ml IHC	Hycult
Anti-C3	Rabbit anti-mouse C3 pAb	Affinity purified	ELISA	2µg/ml	Hycult
Anti-C9	Rabbit anti-rat C9 pAb	Affinity purified	IHC	1/50	In house
Anti-MsE	Rabbit anti-mouse erythrocyte pAb	serum	FC, KA	As defined	In house
BB5.1	Mouse anti-mouse C5 mAb	IgG1	depletion	10mg/ml	Hycult
Anti-C4	Mouse anti-human C4 mAb	IgG1	depletion	10mg/ml	In house
Isotype Control	Rat IgG1	IgG1	IHC	10µg/ml	Caltag
Isotype Control	Rat IgG2a	IgG2a	IHC	5µg/ml 10µg/ml	Caltag
Isotype Control	Rat IgG2b	IgG2b	IHC	5µg/ml 10µg/ml	Caltag
Isotype Control	Rat IgG2c	IgG2c	IHC	10µg/ml	Caltag
Anti-CD4	Rat anti-mouse CD4 mAb	IgG2a	IHC	5µg/ml	BD-bioscience
Moma-2	Rat anti-mouse macrophage mAb	IgG2b	IHC	5µg/ml	Abcam

Table 2.2: FC: flow cytometry, WB: Western blot, IHC: Immunohistochemistry, ELISA: Enzyme-linked immunosorbent assay, KA: Killing assay.

2.3.1. Cell storage

For storage, one T175 flask at ~80% confluency was used. Cells were lifted, washed and re-suspended in freezing solution (10% DMSO (Sigma), 90% FCS (Invitrogen)) and placed in 1ml cryovials (Greiner). Cryovials were placed into Cryo 1°C Freezing Container (Nalgene) and incubated at -80°C for 24 hours. Cryovials were removed from the Container and stored in liquid nitrogen until required. Upon retrieval, cells were thawed quickly, centrifuged (800g, 3 minutes), re-suspended in 30ml media and passaged once *in vitro* prior to inoculation.

2.3.2. Fibrosarcoma cell inoculation

Cells were cultured as described to 70-80% confluency, washed with 0.9% NaCl solution, lifted with Versene (0.5ml, 10 minutes, room temperature), strained through 40µm filter (SC Lab Supplies) and counted. An estimation of cell number was performed using a haemocytometer (MarienField) with cells pre-mixed 1:1 with Trypan blue solution (Fluka). Dead (blue stained) cells were not counted, and cultures were discarded if <90% viable. 10^5 cells were re-suspended per 100µL sterile PBS and injected sub-cutaneously into the flank. Mice were monitored three times per week, with tumour volumes measured as described in section 2.2.

2.3.3. Cloning of fibrosarcoma lines

Freshly excised tumours were disaggregated using forceps, scissors and repetitive syringing. The resulting cell suspension was washed three times (800g, 3 minutes) in 10ml culture media and passed through a 40µm cell strainer (SC Lab Supplies). Heterogeneous fibrosarcoma populations were incubated as described (section 2.3). Media was removed daily, cells washed once with 0.9% NaCl solution (Fresenius Kabi Ltd) to remove cell debris and media replaced. Initially, 20% FCS media was used, reverting to 10% when cultures became established (~ 1 week). Cells were harvested and re-suspended 900 cells/ml in fresh media. Cells were then titrated by 100µl

limiting dilution into a 96 well plate. Wells containing a single, localised cell population were identified, and cells allowed to divide several times before lifting with Versene and re-plating into T_{25} flask in 5ml culture media. This process was repeated 3 times, with populations screened by flow cytometry for uniform expression of CD140a following each cloning step.

2.3.4. Alamar blue proliferation assay

15000 cells were seeded in triplicate into 6 well plates with 1ml culture media. Cells were allowed to adhere for 2 hours before removing media and replacing with 0.5ml media supplemented with 10% (v/v) Alamar Blue solution (AbD Serotec). Plates were incubated at 37°C for 2 hours before 50µL supernatant was removed and placed into a flat-bottomed 96 well plate. Cells were washed using 0.9% NaCl solution (Fresenius-Kabl Ltd) and 1ml culture media replaced. Plates were re-incubated at 37°C. This procedure was repeated at 24 hour intervals. Supernatants in 96 well plates were read using a Fluostar Otima (530 nm excitation, 590 nm emission wavelength).

2.4. Haemolysis assays

2.4.1 Sensitisation of rabbit erythrocytes

Rabbit blood was harvested and mixed with an equal volume Alsever's solution to preserve cells and prevent clotting. Cells were centrifuged (900g, 5 minutes, 4°C) and diluted to 4% v/v in CFD. Polyclonal mouse anti-rabbit erythrocyte Ab was diluted to 0.5% v/v in CFD and incubated 1:1 with erythrocyte suspension for 30 minutes at 37°C. Cells were washed to remove unbound Ab (900g, 5 minutes, 4 °C) and re-suspended to 2% in CFD.

2.4.2. Serum collection

Normal mouse serum was harvested from C57Bl6 mice following sacrifice under Schedule 1 protocol. Blood was collected and clotted on ice for 15 minutes. The resulting clot was spun at 8000g for 10 minutes at 4°C. Upper serum fraction was removed and used immediately or stored at -80°C.

2.4.3. Haemolysis

Mouse serum (50µl) was plated with 50µl CFD in column 1 of a 96-well round bottomed plate and titrated with further CFD via ½ serial dilution. 50µl of antibody coated rabbit erythrocytes were added to each well. Control wells were included in which 50µl dH_2O or CFD was added in place of serum to provide 100% and 0% lysis controls respectively. Duplicate wells were prepared for each serum dilution and control. Following incubation at 37°C for 30 minutes, intact erythrocytes were pelleted by centrifugation (900g, 5 minutes). Flat-bottomed plates were prepared with 100µL dH_2O per well, and 50µL erythrocyte supernatant added per well. Absorbance of the supernatant was read at 415nm using a Dynex MRX II plate reader. Percentage lysis was calculated at each serum dilution according to the following equation:

$$\% \text{ lysis} = (A_{415}^{\text{sample}} - A_{415}^{0\%}) / (A_{415}^{100\%} - A_{415}^{0\%}) \times 100$$

2.4.4. *in vitro* C5 depletion

To assess C-inhibitory effects of BB5.1, sensitised erythrocyte cells were prepared as described in section 2.4.1 and lysed using a range of serum dilutions. The concentration of serum giving 60% lysis was noted. Antibody (BB5.1 or isotype control) was diluted in CFD and 50µl plated at 10µg/ml in duplicate. Further dilutions were prepared by ten 1:1 serial dilutions in CFD. 50µl of the appropriate dilution of mouse serum (to give ~60% lysis) was added and plates shaken briefly to mix. 50µl antibody coated erythrocytes were added per well and haemolysis performed and measured as described above.

2.4.5. *In-vivo* C5 depletion

2.4.5.1. Titration

Monoclonal mouse anti-mouse C5 antibody (BB5.1) in sterile PBS was injected (200µL, 10mg/ml) intra-peritoneally per mouse at 6-day intervals. Serum was harvested regularly via tail tipping and allowed to clot on ice for 20 minutes. The resulting clot was centrifuged (8000g, 5 minutes, 4°C) and serum fraction (top layer) removed. Sera were assayed for C activity via haemolysis assay (as above). Haemolytic activity was plotted and used to determine period over which sera were C5-depleted i.e. non-haemolytic. This was used to select an appropriate *in vivo* dosage for further experiments.

2.4.5.2. Tumour inoculation

To deplete C5, 2 mg BB5.1 or isotype control antibody (mouse anti-human C4) was administered intra-peritoneally on day -1. Fibrosarcoma cells were injected sub-cutaneously (as in section 2.3.2) on day 0 (10^5 cells, 100µL PBS). Repeat antibody doses (2mg) were administered at 6-day intervals.

2.5. Antibody preparation

Antibody producing hybridoma cells were maintained in CELLine 1000 Integra flasks (Integra Biosciences). These flasks comprise a lower (small) and upper (large) compartment separated by a 10kDa semi-permeable membrane. Cells were inoculated at initial density of ~$2x10^7$ cells suspended in 15ml media (15% v/v heat-inactivated low-bovine IgG FCS) (Gibco) in RPMI-1640 (Gibco), 50U penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1% non-essential amino acids (Invitrogen) . 1L RPMI-1640 supplemented with 50U penicillin/streptomycin, 2mM L-glutamine and 1mM sodium pyruvate was added in the upper compartment. To harvest cells, the upper compartment was drained, 20ml medium removed from lower compartment and 1ml cells replaced with 15ml fresh media. Remaining supernatant was spun to pellet

cells (800g, 3 minutes) supernatant removed and frozen at -20°C. This process was repeated as required (~6 day intervals).

2.5.1. Antibody purification

Hybridoma supernatant was thawed and pooled before high speed centrifugation (6000g, 30mins, 4°C) to remove cell debris. The s/n was filtered through a 0.2µm Stericup filter unit (Millipore). An equal volume of saturated ammonium sulphate was added to the s/n and left overnight at 4°C with stirring. Precipitate was collected by centrifugation (6000g, 30mins, 4°C) and re-suspended in AnalaR H_2O until dissolved. Precipitate was collected by centrifugation (6000g, 30min, 4°C) before re-suspension in minimum volume of AnalaR H_2O. The resulting antibody solution was dialysed three times into sterile PBS.

2.5.2. 'Endotoxin-low' antibody purification

Reagents for all buffers used were highest molecular grade available (>99% purity) and were kept sterile. To prepare buffers, solids were weighed in sterile boats in a fume cabinet and were not allowed to contact any other object e.g. a spatula. Prior to protein purification, the AktaPrime protein purifier (GE Life Sciences) and all lines were pre-washed 3 times with 0.5M NaOH to remove all traces of endotoxin. All lines were thoroughly rinsed using PBS running buffer (Gibco) to remove NaOH. The purifier was equilibrated and fitted with an unused 5ml protein G column (Hi-Trap), able to bind ~30mg Ab per purifier run. Pooled tissue culture supernatant containing Ab was thawed, filtered through a 0.2µm Stericup filter unit and passed over the column until saturation i.e. maximum Ab amount bound to column. This volume was determined to be ~40ml per run. To elute protein from the column, 0.1M Glycine (Acros) was prepared using AnalaR H_2O and was acidified to pH 2.5 with HCl (Sigma). Fractions were collected in sterile (pre-packed) Facs tubes (BD-Falcon). Those fractions containing Ab were identified using the absorbance trace via Primeview software and neutralised using 100µl 1M Tris/HCl pH9. The column was cleaned using HCl in AnalaR

H_2O pH1.5, before repeating load cycle. Neutralised Ab fractions were pooled and placed into dialysis tubing pre-boiled in 0.5M NaOH. Ab solution was dialysed twice into sterile PBS (16 hours, 4 °C) using the original PBS packaging and a stirrer pre-washed with 0.5M NaOH. Ab in PBS was aliquoted stored at -20°C until required.

2.5.3. Endotoxin testing

Endotoxin contamination was quantified using the QCL-1000 Chromogenic LAL test (Lonza) according to kit instructions. Screening of 'endotoxin-low' antibody preparations was performed by the Endotoxin screening lab, Medical Microbiology, Cardiff University.

2.5.4. Protein concentration & quantification

Following purification of antibody preparations, solutions were concentrated to 10mg/ml using Amicon pressure ultrafiltration systems, through a 100kDa filter. A Jenway Genove or Nanodrop ND1000 (Labtech) spectrophotometer was first used to set a baseline 'zero' absorbance reading at 280 nm (A_{280nm}) using buffer only (PBS). Ab containing samples were then tested and A_{280nm} reading noted. The Beer-Lambert Law ($A=\epsilon.c.l$) was used to calculate protein concentration whereby the extinction co-efficient (ϵ) for Ab = 1.4 and the length (l) = 1 cm.

2.6. SDS-Polyacryiamide gel electrophoresis (SDS-PAGE)

The Hoefer gel electrophoresis system was used for all experiments. 10µl protein containing samples were boiled for 2 minutes with an equal volume or greater of loading buffer (under reducing or non-reducing conditions). Gels were prepared according to Table 2.5 and submerged in SDS-PAGE running buffer. 20µl protein solution was loaded per well and gels subjected to 200V for 60-75 minutes. Resolved gels were stained or Western blotted as described below. Pre-stained broad range markers were co-loaded to allow size estimation (New England Biolabs).

2.6.1. Cellular lysates

K6 cells were grown until 80% confluent, washed, lifted using Versene and used to make three fibrosarcoma lysates as described in Table 2.4. All steps were performed on ice with resulting lysates centrifuged (15000g, 4 minutes, 4 °C) to remove cell debris. Lysates were made fresh and used immediately.

	1	2	3
Detergent	3% NP40	3% NP40	5% Triton X-100
PMSF	0.001M	0.001M	0.001M
EDTA	10mM	10mM	10mM
SDS	0.05% (w/v)	0.05% (w/v)	0.05% (w/v)
Protease Inhibitor Cocktail (Sigma)	5% (v/v)	5% (v/v)	5% (v/v)
Cells	$20x10^6$ per ml	$10x10^6$ per ml	$10x10^6$ per ml
Incubation	30 mins	60 mins	30 mins

Table 2.3: Cell lysis buffers. Lysates prepared and adjusted to pH 7.4

2.6.2. Coomassie Stain

In order to detect 2-8µg of protein, SDS PAGE gels were immersed in R250 Coomassie blue solution with agitation for 30 minutes following by repeated immersion in destain solution until non-specific staining was removed. Gels were submerged in gel drying buffer (30 minutes) and dried overnight between two sheets of gel drying film (Promega) stretched in a gel drying frame.

2.6.3. Western Blot

Resolved gels were submerged in transfer buffer and blotted (100V, 1 hour) onto 0.2µm nitrocellulose (VWR International) sandwiched between blotting paper and sponges pre-soaked in transfer buffer and placed in a cassette. Following transfer, nitrocellulose was removed and blocked in PBS-milk (1 hour, room temperature) before incubation with primary antibody (see Table

2.2) (14 hours, 4°C). Blots were washed 3 times (10 mins, room temperature) in PBS-Tween solution before re-blocking with PBS-milk (30 minutes, room temperature). Blots were stained with 1/1000 v/v donkey anti-rat IgG-HRPO (Jackson Immunoresearch) (1 hour, room temperature) before washing three times with PBS-Tween solution and once with PBS. Bound Ab was visualised using Enhanced Chemiluminesce kit (GE Healthcare), subjected to light sensitive hyperfilm (GE Healthcare) and developed using Compact X-2 X-Ograph developer machine.

Percentage gel	**5% (stacking)**	**7.5% (resolving)**	**10%**	**12.5%**
Stacking gel Buffer (ml) pH6.8	2.4	-	-	-
Resolving gel Buffer (ml) pH8.8	-	7.5	7.5	7.5
40% Acrylamide (ml)	1.3	5.6	7.5	9
10% Ammonium Persulphate(APS) (µl)	100	300	300	300
dH_20 (ml)	6.1	16.4	14.5	13.0
TEMED (µl)	10	30	30	30

Table 2.4: Gel compositions for SDS-PAGE. Acrylamide, APS and Temed all sourced from Sigma-Aldrich.

2.7. Flow Cytometry

2.7.1. Cell staining

Cells were characterised for protein expression using a 2 step staining process. Cells were lifted, strained and counted as described in Section 2.3.2. Cells were re-suspended to 10^6/ml in FACS buffer and 100µl incubated with the same volume of primary Ab (60 minutes, 4°C) in a 96-well plate (Abs used are detailed in Table 2.2). Cells were pelleted (800g, 4 minutes) and washed three times in FACS buffer. 100µl re-suspended cells were incubated 1:1 with 1/100 v/v species-specific R-Phycoerythrin labelled secondary antibody (60 minutes, 4°C) in 96-well plate (Table 2.2, Jackson Immunoresearch). Cells were pelleted (800g, 4 minutes) and washed three times in FACS buffer.

2.7.2. Flow cytometric analysis

Cell events were collected using a FACSCalibur and analysed using CellQuest Pro software (BD Biosciences). A minimum of 15000 'events' were collected per sample, and 10^6 erythrocytes were analysed for controls. Data were gated to remove cellular debris and analysed for fluorescence in the FL-2 channel using Summit v3.1 (Dakocytomation).

Antibody	Conjugate	Application	Dilution	Source
Donkey anti-rat IgG	RPE HRPO	FC WB	1/100 1/1000	Jackson
Donkey anti-rabbit IgG	RPE	FC	1/100	Jackson
Goat anti-rabbit IgG	HRPO	ELISA	1/1000	Jackson
Donkey anti-rat IgG	Alexa-fluor-594	IHC	1/200	Molecular Probes Inc.
Goat anti-rabbit IgG	Alexa-fluor-594	IHC	1/200	Molecular Probes Inc.

Table 2.5: FC: flow cytometry, WB: Western blot, IHC: Immunohistochemistry, ELISA: Enzyme-linked immunosorbent assay.

2.7.3. Killing assay: propidium iodide

Cells were grown until 80% confluent, lifted using 0.5ml Versene, washed and re-suspended at $2x10^6$/ml in CFD. 50µl polyclonal rabbit anti-mouse erythrocyte antibody (in CFD) was added to 50µl cells with final antiserum concentrations from 10% to 0.5% v/v in a 96-well plate. Cells were incubated with heat inactivated (30minutes, 56°C) antiserum (45 minutes, 4°C) before washing twice in CFD. Cells were transferred to FACS tubes after re-suspension in 100µL of a range of fresh mouse serum (100% to 2% final concentrations v/v in CFD). After 1 hour at 37°C, 200µl of 2µl/ml propidium iodide (PI) was added to FACS tubes and fluorescence measured by flow cytometry as described above. PI is an intercalating agent which penetrates cells with disrupted membranes (i.e. dead/dying) and binds to DNA. PI emits a fluorescent signal which can be used to differentiate healthy cells (non-fluorescent) from lysed cells (PI fluorescent).

2.7.4. Killing assay: calcein-AM loading

Cell viability can be assayed by pre-loading with a compound and quantifying release of the compound following sensitisation and treatment with serum. For this assay, the cell permeable compound calcein acetoxymethyl ester (calcein-AM) was incubated with cells and is readily taken up to 'load' cells with the non-fluorescent dye. Intracellular esterases cleave the compound by hydrolysis to yield the fluorescent dye calcein. Release of free calcein by dead cells can be detected and used to quantify cell death following sensitisation and treatment with C. Intact cells can then be lysed with detergent allowing calculation of the proportion of cells lysed through C as a percentage of the total population.

Cells were seeded to 30-40% and grown overnight to obtain 50-60% confluence in 24 well plates. 50µg calcein-AM (Molecular Probes) was dissolved in 50µl DMSO (Sigma) + 50µl methanol. 25µl 2M KOH (H_2O) was added and the calcein solution (1 hour, room temperature), diluted to 2µg/ml in 5% FCS-RPMI and the solution was adjusted to pH7. Cells were loaded by

incubation with 200µl per well calcein (2µg/ml, 5% FCS-RPMI) for 1 hour at 37°C and washed with 1% BSA/ CFD. Cells were sensitised for 45 minutes at 4°C with 7% v/v heat inactivated (56°C, 35 minutes) pAb in 175µL BSA/CFD. Cells were washed before incubation (37°C, 30 minutes) with 200µl of a range of fresh mouse serum concentrations (100% to 2% v/v in BSA/CFD). This and subsequent steps were performed in the dark to avoid quenching of fluorescent signal. Supernatants were transferred to a 96-well plate. Remaining calcein was released from non-lysed cells using 200µl 0.5% Triton X-100 (Sigma-Aldrich)/CFD v/v for 15 minutes and s/n was transferred to a 96-well plate. Fluorescence was read using a Fluostar Optima (excitation 485nm, emission 530nm) and plotted using Graphpad Prism. % C-mediated lysis was calculated according to the below formula whereby $A^{0\%}$ refers to background calcein release in wells untreated by C or detergent:

$$\% \text{ lysis} = (A^{\text{C-mediated}} - A^{0\%}) / (A^{\text{C-mediated + detergent-mediated}} - A^{0\%}) \times 100$$

2.7.5. Complement C3b/iC3b deposition assay

K6 cells were grown until 80% confluent, lifted as described in Section 2.3.2 and re-suspended at 10^6/ml. 100µl cells were sensitised in 96-well plates using 2% v/v heat inactivated (30minutes, 56°C) rabbit anti-mouse erythrocyte pAb in CFD (45 minutes, 4°C). Cells were washed in CFD and 100µl cells incubated (30 minutes, 37 °C) with a range of fresh mouse serum (final concentrations 50%-0.5% v/v). Cells were washed three times (800g, 3 minutes) into FACS buffer. Mouse C3b/iC3b deposition was detected by flow cytometry using 10µg/ml 11H9 rat anti-mouse C3 mAb by incubation at 4°C for 45 minutes. Cells were washed three times (800g, 3 minutes) before incubation with 1/100 (v/v) donkey anti-rat IgG-RPE (cross-adsorbed against rabbit Ig to prevent binding to sensitising pAb). Shift in fluorescence was measured using a FACSCaliber and analysed using Summit v3.1 (Dakocytomation).

2.8. Immunohistochemistry

Excised tumours were snap frozen in -20°C iso-pentyl alcohol and stored at -80°C. Tumour sections of 10μm thickness were collected using a Thermo Shandon cryotome and fixed in acetone (15 minutes, 4°C). Sections were blocked using 2% BSA in PBS (w/v) and stained (1 hour, room temperature) for C deposition / regulator expression using in-house monoclonal antibodies (Table 2.1). Appropriate isotype control antibodies were included alongside each staining procedure. Following incubation with antibody, sections were washed 3 times in PBS to remove non-specific binding. Sections were re-blocked for 30 minutes with 2% BSA in PBS. Fluorescent (Alexa-594 conjugated) secondary antibodies (Table 2.3, Molecular Probes Inc.) were used (1 hour, room temperature) to detect binding of primary Abs. Fluorescent images were taken using a Leica microscope and Hamamatsu C4742-95 digital camera. Images were de-compressed using Velocity.

2.8.1. Fluorescence quantitation

Images were captured from non-overlapping random positions per tumour section and assessed for C3b/iC3b deposition, C9/MAC deposition or $CD4^+$ infiltrating cells. A total of 8 tumours was selected per genotype, with 6 frames recorded from each tumour section.

2.8.1.1. C3b / C9 deposition

Images were loaded into Image-Pro Analyser 6.3 software. This software allows determination of fluorescence within an image by assessing fluorescent signal relative to a user-defined standard. A background threshold for fluorescence was set to recognise areas stained positive by eye and maintained for all photos analysed. Area of field stained positive was measured automatically and expressed relative to the entire frame area. % fluorescence was then recorded and analysed using Graphpad Prism.

2.8.1.2. $CD4^+$ cell infiltration

Images were captured from non-overlapping random positions per tumour section. Sections were counted blind to avoid bias, and verified by an independent blinded observer. Cells were visible as a defined ring of fluorescence. The total number of single cells stained positive for CD4 antigen were recorded and analysed using Graphpad Prism.

2.9. Quantitative real-time PCR analysis

2.9.1. RNA Extraction

Total RNA was extracted and purified from fibrosarcoma cell lines using the GenElute Mammalian Total RNA Miniprep kit according to the manufacturer's instructions (Sigma-Aldrich). Additionally, RNA from 1g homogenised fresh (WT) liver sample was extracted and purified using the same kit. All protocols involving RNA extraction were carried out using DEPC-treated H_2O, pipette tips and tubes to prevent RNA degradation. Briefly, cells were lysed in guanidine thiocyanate and β-mercaptoethanol to release RNA and inactivate RNases before centrifugation (12000g, 10 minutes, 4°C) through a filtration column to remove cell debris and DNA. RNA was then precipitated using isopropanol and spun (12000g, 10 minutes, 4°C) to produce an RNA pellet. RNA was re-suspended and washed twice in 70% ethanol (8000g, 5 minutes, 4°C) before re-suspending in DEPC-H_2O. RNA concentration was assessed using Nanodrop ND1000 (Labtech).

2.9.2. qPCR

1µg cDNA from each cell line was prepared from RNA using random hexamers and multiscribe reverse transcriptase according to the manufacturer's instructions (Applied Biosystems, Warrington, U.K.). The Platinum SYBR Green qPCR Super-Mix-UDG kit was used for real-time qPCR including *Taq* DNA polymerase. Primer RNA strands were designed specifically to hybridise to genes of interest (mouse C3, CD55, CD59 and β-

actin). Forward and reverse primers were included as described in Table 2.6 and PCR conditions were as follows:

12.5µL	Platinum SYBR Green qPCR Supermix
0.5µL	Forward primer (10µM)
0.5µL	Reverse primer(10µM)
50ng	cDNA template
6.5µL	DEPC-treated H_2O

PCR conditions:

	Activation:	95°C	3 minutes
Repeat 40 cycles	Denaturation:	95°C	15 seconds
	Annealing:	58°C	30 seconds

Reactions were carried out in 48 well plates, with each cell line assessed in triplicate. qPCR was performed on the ABI PRISM 7000 (Applied Biosystems) and analysed using the sequence detection system software version 1.9 (Applied Biosystems). SYBR Green uptake was normalised using β-actin expression levels to control for differences in cDNA loading. Results were calculated and expressed relative to results from lines deficient in C3, CD55 or CD59.

	Primer sequence
B-actin forward	ACGGCCAGGTCATCACTATTG
B-actin reverse	AGTTTCATGGATGCCACAGGAT
CD55 forward	TGTGATGCTCTTACTCACTGG
CD55 reverse	GTATCCATTCTTCCTGGACATTC
CD59a forward	GCCGGAATGCAAGTGTATCA
CD59a reverse	GTCCCCAGCAATGGTGTCTT
C3 forward	AGAAGACTGCCTGACCTTCA
C3 reverse	TTTTCTCCCCAGAGGTCAGA

Table 2.6: Forward and reverse primer sequences for real-time qPCR.

2.10. ELISA

96-well ELISA plates (MP Biochemicals) were coated with 90µl rat anti-mouse C3 mAb (11H9) at 10µg/ml in ELISA coating buffer (1 hour, 37°C). Plates were then washed 3 times with 150µl 0.1% Tween-20 (Acros Organics)/PBS. Plates were then blocked with 5% milk/PBS (w/v) (1 hour, 37°C) and incubated with 90µl purified C3 from WT mice or tissue culture supernatant in triplicate. For some assays, supernatant was concentrated using 0.22µm centrifugal concentrator (Millipore). After washing, 90µl polyclonal rabbit anti-mouse C3 was used at 2µg/ml to detect bound C3 (1 hour, 37°C) followed by washing and detection using HRPO-conjugated 1/1000 goat anti-rabbit IgG (1 hour, 37°C). Plates were washed and signal developed using OPD solution and stopped by addition of 45µl 10% H_2SO_4. Absorbance was read at 490nm in Dynex Technologies MRXII.

3. Characterisation of a WT fibrosarcoma line for *in vivo* inoculation

3.1. Introduction

The aims of experiments described in this Chapter were to characterise the *in vitro* behaviour and confirm *in vivo* growth of a fibroblast cell line available in house. This was done in order to later use the cell line to investigate the impact of the C system on tumour growth and progression. As described in Chapter 1, tumours can be induced by administration of the chemical carcinogen 3-MCA. Injection of this substance results in recruitment and subsequent mutation of fibroblasts at the site of injury. A progressively growing tumour is formed which may be excised and cultured *in vitro*. A cell line derived *in vitro* from a 3-MCA induced fibrosarcoma was obtained from Dr Maries van den Broek (University Hospital, Zurich). This cell line, named K6, was found to grow aggressively when re-inoculated into WT mice (G.J.Betts personal communication, November 2005). No work using this cell line has previously been published, however, it was surmised that inoculation of these cells into various hosts could be used to identify differences in tumour progression between WT and C-deficient host mice. As such, this cell line is expected to provide a robust model for the growth of established tumours *in vivo*.

In order to achieve this goal, it was first considered important to perform a detailed characterisation of this cell line with respect to relevant *in vitro* properties and *in vivo* growth rates. Thus, this Chapter describes assessment of fibrosarcoma phenotype and expression of 3 important CRegs by these cells. Such properties were examined via the use of monoclonal antibodies (mAbs) against CD140a (a fibroblast marker), CD55, CD59 and Crry (CRegs) proteins. Expression of CReg provides effective protection from C-mediated lysis and may be important for assessment of *in vivo* survival and progression.

Additionally, the possibility that these cells synthesise and secrete C3 *in vitro* was investigated. This hypothesis is based on previous reports detailing co-secretion of melanoma-derived C3 along with a C3 cleaving enzyme (Jean et

al. 1997), and C3 secretion by human gastric cancer derived cells (Kitano and Kitamura 1993). Fibroblast cells have also been shown to produce local C3 following cytokine stimulation (e.g. IL-1, IL-6, IL-13) suggesting that fibrosarcoma cells may be good candidates to investigate the possibility and effects of C3 secretion by transformed cells (Katz, Revel and Strunk 1989).

3.2. Results

3.2.1. Characterisation of K6 line for fibrosarcoma phenotype

K6 cells cultured *in vitro* were found to be extremely adherent and showed similar morphology to normal fibroblasts. In order to further characterise these cells, the CD140a antigen was considered an appropriate marker to confirm the fibroblastic origin of the K6 line. This 170kDa antigen is also known as Platelet Derived Growth Factor Receptor α (PDGFRα, CD140a), is expressed on fibroblasts and implicated in cell migration (Yu et al. 2001). A rat mAb specific for this protein was used to detect expression via flow cytometry and visualised using an RPE-conjugated donkey anti-rat secondary Ab.

Flow cytometry data shown in Figure 3.1 indicates a relatively homogeneous population as evidenced by a defined population in terms of forward and side scatter (Panel A). Panel B shows fluorescence exhibited upon staining for CD140a expression. Cells were gated to fall within the region marked R1 in Panel A. Gating to this region included approximately 90% of the entire population and excluded dead/dying cells which may distort fluorescence measurements. Displayed in panel B is a control population (shaded area) stained with secondary Ab only, while the test population treated with anti-CD140a Ab is also shown and caused an increase in fluorescence. This indicates detectable levels of CD140a expression on K6 cells. Data shown is representative of 5 experiments performed at different times on K6 populations. In each case, a shift in median fluorescence intensity (MFI) was recorded and found to range between 15-22 MFI units. This value was calculated in reference to the control population not exhibiting fluorescence and provided a measure of relative protein expression.

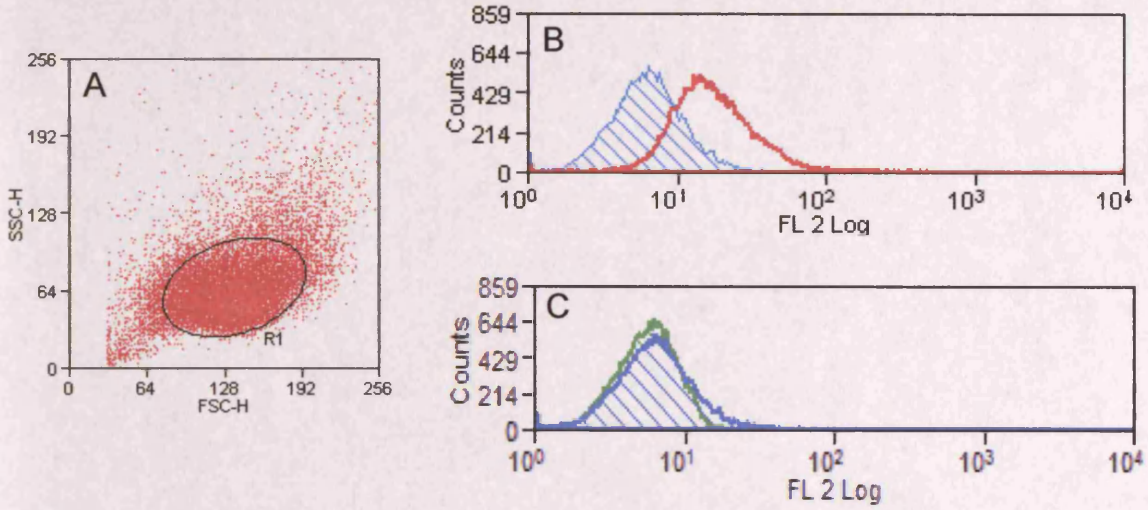


Figure 3.1: Expression of CD140a on cultured K6 cells.
K6 cells were lifted, dis-aggregated and stained for CD140a expression using a two step staining protocol. Apa5 rat anti-mouse CD140a mAb was used and detected by donkey anti-Rat IgG-RPE. Panel A depicts forward and side scatter gated to region 1 (R1). Panel B depicts shift in fluorescence following staining for CD140a. Blue shaded area represents control cells, red line indicates shift in fluorescence following staining for CD140a antigen. Panel C depicts control (untreated cells) and those treated with isotype control Ab.15000 cells were analysed. Data shown are representative of 5 separate experiments.

3.2.2. CReg characterisation of K6 cells

3.2.2.1. Flow cytometry

CReg expression by tumour cells is a key mechanism by which cells are protected from C-mediated lysis and are important considerations when investigating the relationship between C and fibrosarcoma lines. Protein expression levels were analysed using flow cytometry. A rat mAb specific for Crry (5D5) was used and detected a significant increase in fluorescence when compared to a control population. However, rat mAbs specific for CD55 (MD1) and CD59 (Mel4) did not bind to cells at detectable levels with no shift in fluorescence observed. These data are shown in Figure 3.2 and suggest that high levels of Crry, but no CD55 or CD59 are expressed on K6 cells. As previously, these data are representative of 5 independent experiments. In order to exclude a problem with Ab binding and detection in the assay, mouse erythrocyte cells were also included as a positive control. Non-nucleated cells (e.g. erythrocytes) are prone to C-mediated lysis and are known to express high levels of CReg (Miwa et al. 2002b). As shown in Figure 3.3, a clear shift in fluorescence was observed following staining for Crry, CD55 and CD59 on erythrocytes. These data indicate that the lack of CD55 and CD59 on K6 cells is a genuine observation and is due to lack of expression and not a problem with the detection method.

Flow cytometry analysis of these cells was performed on several occasions using freshly thawed cells passaged only once or twice *in vitro*. Collated data from 5 separate experiments are summarised in Figure 3.4. This Figure shows little variation between experiments and confirms a consistent lack of CD55 and CD59 but high expression of Crry on K6 cells. The CD140a protein was also detected on all populations analysed.

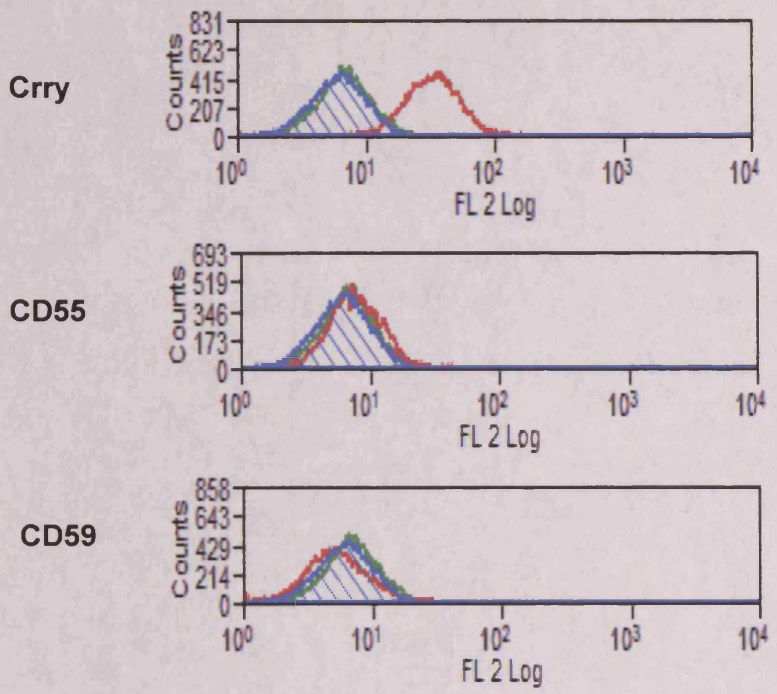


Figure 3.2: Expression of CReg by K6 cells.
K6 cells were lifted, disaggregated and stained for CReg expression using a two step staining protocol. 5D5, MD1 and Mel4 rat anti-mouse Crry, CD55 and CD59 respectively were used and detected by donkey anti-Rat IgG-RPE (red line). Control (unstained) cell populations are shown in blue, with isotype control treated populations in green. 15000 cells were analysed. Data shown are representative of 5 separate experiments.

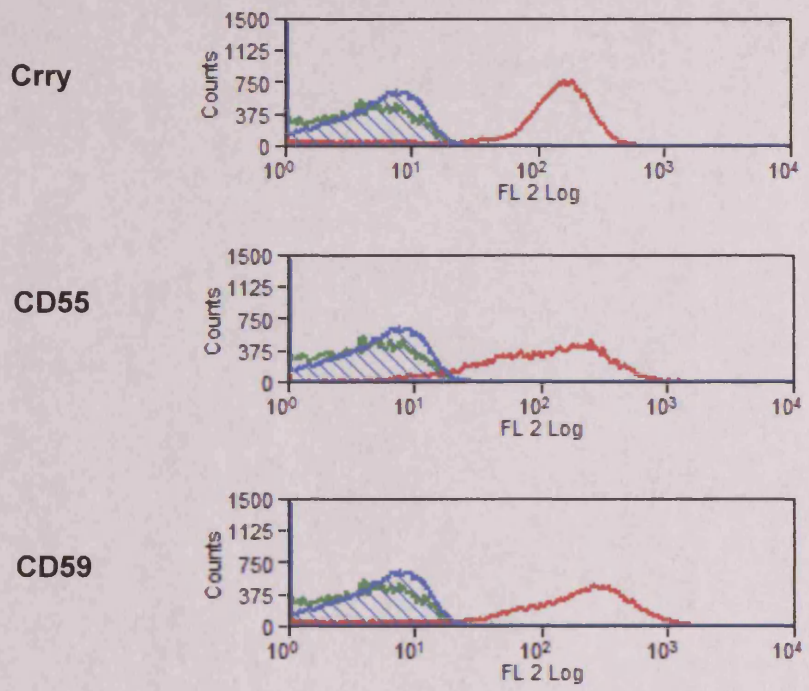


Figure 3.3: Expression of CReg by mouse erythrocytes
Mouse erythrocyte cells were stained for CReg expression using a two step staining protocol. 5D5, MD1 and Mel4 rat anti-mouse Crry, CD55 and CD59 respectively were used and detected by donkey anti-Rat IgG-RPE (red line). Control (unstained) cell populations are shown in blue, with isotype control treated populations in green.10^5 cells were analysed. Data shown are representative of 5 separate experiments.

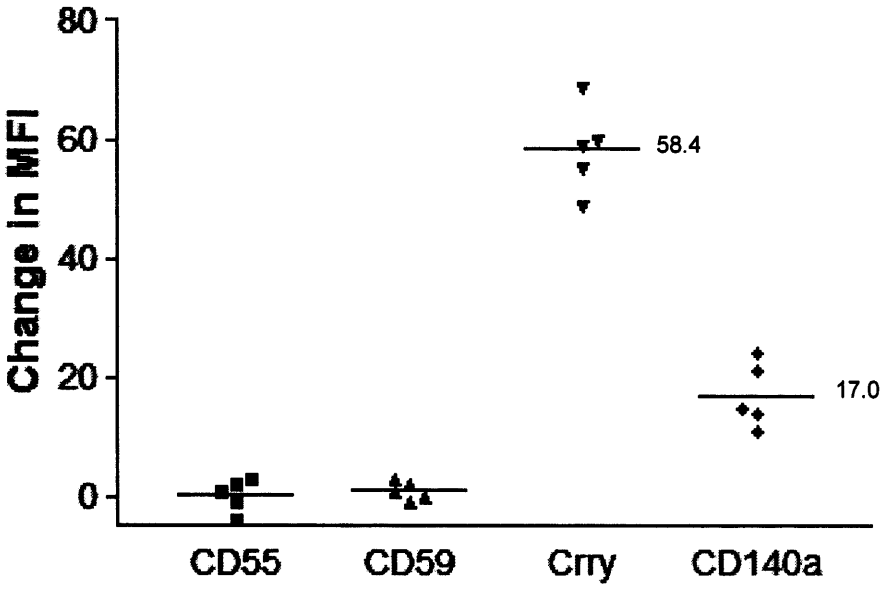


Figure 3.4: Analysis of CReg expression in K6 populations.
K6 populations were stained for CReg. The median shift in fluorescence was calculated following comparison to control cells treated with secondary Ab only. Five different K6 populations were analysed and changes in fluorescence plotted. Figures adjacent to bars represent the mean shift in MFI value.

3.2.2.2. Western blotting

An alternative method for detection of the above proteins is Western Blotting. This method allows detection of proteins in denatured i.e. non-native states as well as allowing estimation of protein molecular weights by reference to markers of known weight. Initial attempts to obtain working lysates proved unsuccessful due to protease digestion of proteins. Considerable optimisation of cell number, lysis protocol and inclusion of protease inhibitors was required as detailed in Chapter 2. K6 lysates were loaded onto 10% polyacrylamide gels and separated by electrophoresis. Resulting gels were blotted onto nitrocellulose and probed with rat mAbs as described above. A peroxidise conjugated donkey anti-rat IgG secondary Ab was used to detect bound Ab and visualised via chemiluminescence. K6 lysates were freshly prepared immediately prior to blotting with differing lysis conditions used to generate 3 lysates from the same population. All three lysates were loaded to control for problems with protein degradation in these samples. Additionally, mouse erythrocyte lysates were included as a positive control for CReg presence.

Figure 3.5 shows presence of CD140a in each of 3 K6 lysates tested (blot A, lanes 1-3). The observed bands for each lysate resolved at 160-170kDa which corresponds to the expected size of the protein. As expected, CD140a was not found in the mouse erythrocyte lysate (lane 4) with no band visible. Data shown in blot B represents identical lysates stained with secondary Ab only. This controlled for non-specific binding of Ab with no bands visible on any blot stained with secondary Ab alone.

Figure 3.6 shows that K6 lysates did not contain detectable levels of CD55, as evidenced by the lack of visible bands in lanes 1-3. CD55 was detected in mouse erythrocyte lysates with a strong band visible at ~60kDa. Similarly, CD59 was not present in fibrosarcoma lysates (Figure 3.7). No bands were visible in lanes 1-3 whereas a band corresponding to ~15kDa was detected in the erythrocyte lysate (lane 4). Figure 3.8 shows the presence of Crry in K6 and erythrocyte lysates with each band corresponding to a molecular weight

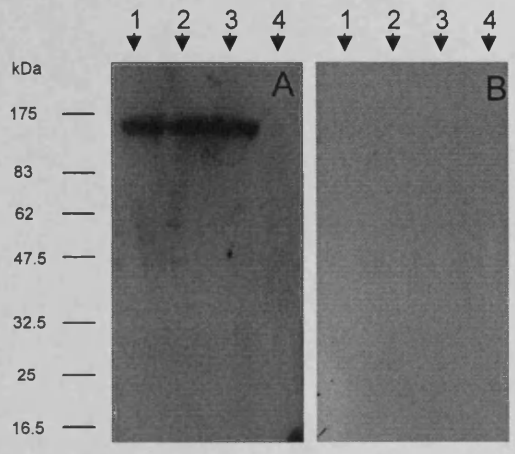


Figure 3.5: Western blot analysis of CD140a in K6 cell lysates
K6 lysates were loaded onto a 10% gel and resolved. The gel was Western blotted onto nitrocellulose and probed with Apa5 rat anti-mouse CD140a. Bound mAb was detected using donkey anti-rat IgG-HRPO. Lanes 1-3 were loaded with K6 lysates prepared in different conditions as described in Chapter 2. Lane 4 was loaded with a mouse erythrocyte lysate. Panel B was not treated with primary antibody and controlled for Ab specificity.

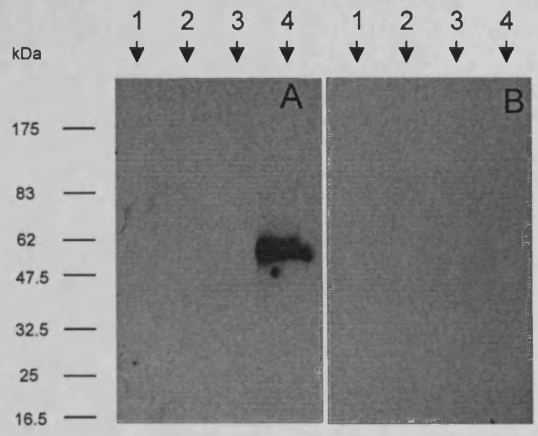


Figure 3.6: Western blot analysis of CD55 in K6 cell lysates
K6 lysates were loaded onto a 10% gel and resolved. The gel was Western blotted onto nitrocellulose and probed with MD1 rat anti-mouse CD55. Bound mAb was detected using donkey anti-rat IgG-HRPO. Lanes 1-3 were loaded with K6 lysates prepared in different conditions. Lane 4 was loaded with a mouse erythrocyte lysate. Panel B was not treated with primary antibody and controlled for Ab specificity.

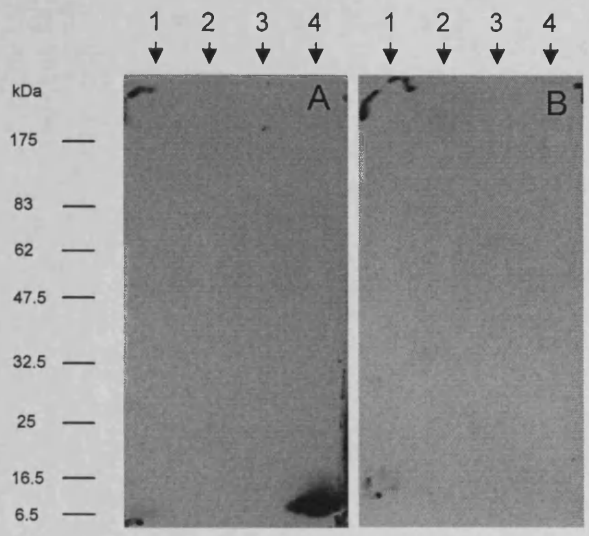


Figure 3.7: Western blot analysis of CD59 in K6 cell lysates
K6 lysates were loaded onto a 10% gel and resolved. The gel was Western blotted onto nitrocellulose and probed with Mel-4 rat anti-mouse CD59. Bound mAb was detected using donkey anti-rat IgG-HRPO. Lanes 1-3 were loaded with K6 lysates prepared in different conditions. Lane 4 was loaded with a mouse erythrocyte lysate. Panel B was not treated with primary antibody and controlled for Ab specificity.

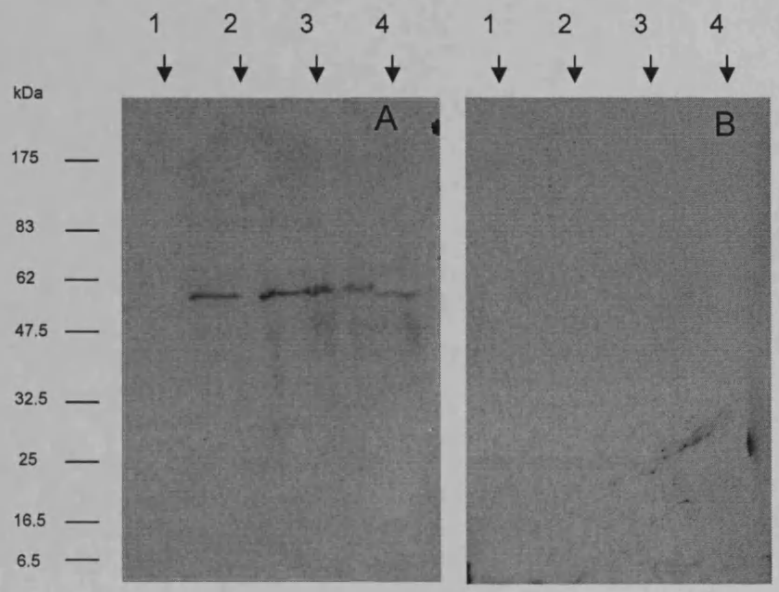


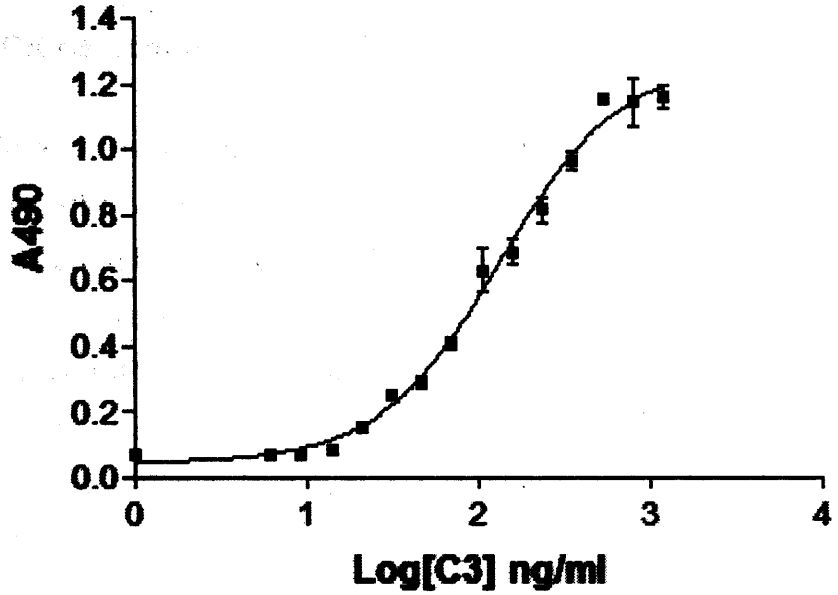
Figure 3.8: Western blot analysis of Crry in K6 cell lysates
K6 lysates were loaded onto a 10% gel and resolved. The gel was Western blotted onto nitrocellulose and probed with 5D5 rat anti-mouse Crry. Bound mAb was detected using donkey anti-rat IgG-HRPO. Lanes 1-3 were loaded with K6 lysates prepared in different conditions. Lane 4 was loaded with a mouse erythrocyte lysate. Panel B was not treated with primary antibody and controlled for Ab specificity.

of ~60kDa. Crry was missing in lane 1, suggesting protein degradation in this lysate. The presence of Crry in lysates 2 and 3 was sufficient to prove that Crry is present in the analysed cell population. In each of Figures 3.5-3.8, the control blot showed no chemiluminescent signal confirming the specificity of the primary Abs.

3.2.3. Estimation of C3 secretion by K6 cells

As described above, cells of fibroblast origin are known to secrete certain C components *in vivo*, an effect that may lead to enhanced tumour progression. Production and/or secretion of the central C component C3 has been reported by tumour cells and may be an important feature in terms of fibrosarcoma proliferation. An Enzyme-Linked ImmunoSorbent Assay (ELISA) was therefore used to assess and quantify C3 secretion by K6 cells into tissue culture media.

A rat anti-mouse C3/C3b/iC3b mAb was used to detect C3 secreted by confluent K6 populations into culture supernatant (s/n). Cells were cultured to ~40% confluence, fresh media added and incubated for 16 hours. S/n was removed from each population (approx $5x10^6$ cells), spun to remove cellular debris and assayed in triplicate for C3/C3b/iC3b presence using a plate-bound rat monoclonal Ab specific for these proteins. Bound protein was detected using a rabbit polyclonal anti mouse C3 Ab and a peroxidase conjugated donkey anti-rabbit IgG Ab. A range of known C3 concentrations was assayed on the same plate comprising a titration of mouse C3 concentrations, purified from WT sera. These known concentrations were used to create a standard curve against which s/n readings could be compared. These data are shown in Figure 3.9 and allow estimation of C3 concentrations in the range 20-360ng/ml. However, absorbance values indicated in Figure 3.9 for a pool of three independently maintained K6 lines correspond to concentrations below the threshold (20ng/ml) for detection in this assay. Consequently s/n from each of the populations were pooled and concentrated 10-fold prior to re-assay, again no C3 was detected (data not shown).



	A490
1	0.049
2	0.055
3	0.051

Figure 3.9: ELISA detection of C3 secretion by K6 cells.
A 96 well plate was coated with a monoclonal Rat anti-mouse C3 antibody and incubated with a serial dilution of known C3 concentrations. Plates were washed before incubation with polyclonal Rb anti-mouse C3. Bound antibody was detected using HRPO-conjugated anti-rabbit IgG and visualised using an OPD preparation. Dilutions of C3 were assayed in triplicate. Values shown adjacent to the graph represent absorbance values following incubation with tissue culture s/n from a K6 population. Data shown are representative of 2 separate experiments.

3.2.4. RT-PCR detection of CD55, CD59 and C3 mRNA

Previous data shown above demonstrate a lack of CD55 and CD59 expressed on cells, while no C3/C3b/iC3b could be detected in tissue culture s/n following incubation with cells. Whilst these data suggest a lack of protein expression or secretion, an alternative method, RT-PCR, can provide a measurement of mRNA transcription *in vitro*. CD55, CD59 and C3 proteins may be transcribed at very low levels or be stored intra-cellularly resulting in the observed result above. However, RT-PCR is a highly sensitive technique and may identify mRNA transcription in the absence of protein expression. mRNA was obtained and reverse transcribed to cDNA prior to amplification and analysed using Platinum SYBR Green qPCR SuperMix (Invitrogen). Primers specific for CD55, CD59 and C3 (method described fully in Chapter 2) were used to assess mRNA content associated with these genes. Figure 3.10 shows negligible amplification of CD55, CD59 or C3 mRNA from K6 cells. Hepatocyte cells were included as a control and showed significant amplification for each protein. These data showed that negligible mRNA for CD55, CD59 or C3 was present in K6 cells *in vitro* and support previous findings described in this Chapter.

3.2.5. *in vivo* growth of K6 fibrosarcomas

Established tumour cell populations can provide valuable models of *in vivo* tumour growth profiles with growth dependent on numerous factors including nature/type of cells, site of injection and number of cells inoculated. In order to identify an appropriate dose of K6 cells, initial experiments were performed using varying cell numbers. Figure 3.11 shows tumour growth in male WT animals as measured following inoculation of 10^4, 10^5 and 10^6 cells respectively. Tumours grew in each group with 100% tumour take in the two groups inoculated with higher doses of K6. Tumours appeared to grow at comparable rates independent of the initial number of K6 cells administered. However, while 100% tumour incidence was observed when 10^5 or 10^6 cells were inoculated, only half of the mice developed tumours following inoculation

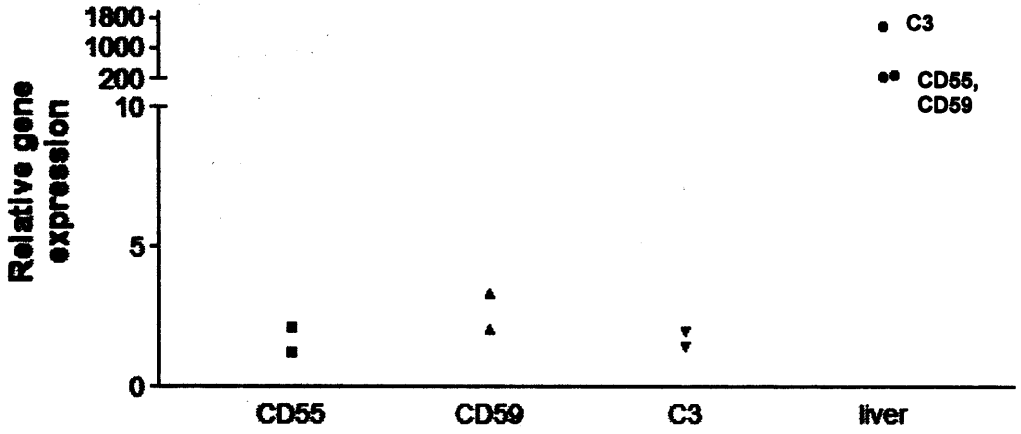


Figure 3.10: RT-PCR analysis of CD55, CD59 and C3 mRNA in K6 cells
mRNA was extracted from K6 cell populations or fresh liver sample. RNA was converted to cDNA via reverse transcription. Primer sequences specific for mouse CD55, CD59, C3 and β-actin were used to amplify sequences in presence of Sybr-Green fluorescent dye. Fluorescence levels per cycle were recorded and used to calculate C(t) values for each protein per cell line. Reference gene data (β-actin) was deducted and values expressed relevant to a population deficient in relevant gene.

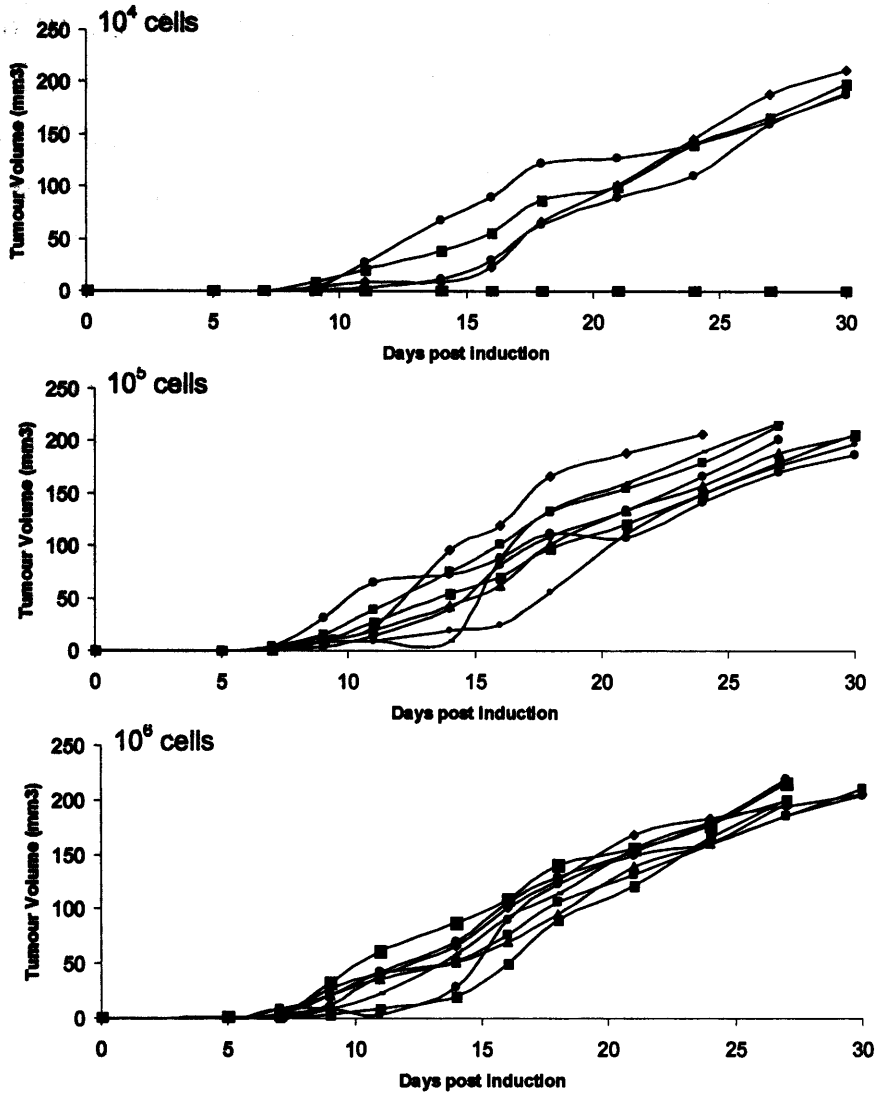


Figure 3.11: *In vivo* tumour incidence following K6 cell inoculation into WT mice. Inoculation of K6 cells into male WT mice. Mice were injected on day 1 with varying doses of tumour cells suspended in 100µL PBS. Number of cells inoculated is displayed next to each curve. Injections were made on the flank and mice monitored regularly with tumour sizes measured. Each line is representative of one individual mouse. Mice were sacrificed when tumour reached 200mm^3. Each group consists of pooled data (n=8) from 2 separate experiments (n=4).

of 10^4 cells. The observation that 100% incidence was recorded following inoculation with 10^5 and 10^6 cells suggests that a threshold for incidence may exist whereby tumour cell numbers are high enough to form a progressively growing tumour in the vast majority of mice injected. Inoculation of 10^5 K6 cells was selected for further experiments detailed in Chapter 4.

3.2.6. Discussion

The aim of the work described in this Chapter was to characterise a fibrosarcoma cell line and assess suitability of this line to investigate the roles of specific C components on tumour growth in mice. Additionally, data obtained through these experiments were used to guide further experiments involving the K6 cell line and described in subsequent Chapters. The experiments performed confirmed that the cells were fibrosarcomas (CD140a – positive) and that they grew progressively in immunocompetent animals. An inoculum of 10^5 K6 cells ensured tumour take in WT mice. Additionally, knowledge of certain cell characteristics was considered important for this project. As such, expression of the membrane CRegs Crry, CD55 and CD59 along with secretion of key C component C3 was assessed.

Flow cytometric staining of K6 revealed unexpected results regarding CReg expression. The presence of Crry but not CD55 or CD59 was confirmed for these cells. This observation is somewhat surprising given the propensity of many tumour types to over-express CReg (reviewed by Fishelson, 2003), and previous reports of CD55 and CD59 expression on rodent fibroblasts (Powell et al 1997, Hinchliffe et al. 1998). However, Crry alone is a potent regulator and is the most important CReg on mouse erythrocytes with deficiency causing spontaneous haemolysis (Miwa et al. 2002b). Deficiency of CD55 and CD59 in the same study had lesser effects with erythrocytes deficient in either of these regulators shown to have a normal lifespan. Whilst K6 cells were shown to be negative for CD55 and CD59 expression *in vitro*, it remains possible that CReg are up-regulated *in vivo* in response to C-attack with numerous cytokines associated with up-regulation of CReg in humans (Takeuchi et al. 2001, Spiller et al. 2000), while CD55 expression may be

induced in mouse endothelial cells by incubation with TNFα (Ahmad et al, 2003).

Western blotting was also used to examine presence of CReg in these cells. This technique provides an estimate of protein molecular weight by comparison to known standards loaded alongside fibrosarcoma and control lysates and confirms data obtained by flow cytometry. This can be an important tool for identifying differential glycosylation patterns associated with certain tumour types (Nakagawa et al. 2001). In these cases, protein bands observed resolved at identical sizes to those derived from erythrocyte lysates indicating similar glycosylation in tumour and red blood cells. An additional advantage of assaying total cell lysates is the ability to detect intra-cellular protein stores i.e. non-surface expressed proteins which would not be detected by flow cytometry as described above. Considerable difficulties were encountered in attaining working lysates. Proteins within resulting lysates were rapidly degraded and protein content lost. Optimised conditions and a 'cocktail' of protease inhibitors were required, with all steps performed on ice. Resulting lysates were effective if used immediately, but no protein could be detected following storage at -80°C. One lysate tested negative for Crry (Figure 3.8), demonstrating the vulnerability of these lysates to degradation. Western Blotting experiments performed in this Chapter confirmed the presence of Crry and CD140a, and the absence of CD55 and CD59 in K6 total cell lysates. These findings substantiate previous findings via flow cytometry.

An important characteristic to consider is the secretion of C3 observed in some tumour cells (Kitano and Kitamura 1993). Additionally, co-secretion of C3 with a C3 cleaving enzyme has been observed in human melanoma cells with secretion shown to correlate with high metastatic potential (Jean et al. 1997). C3 secreted by transformed cells may contribute to the host anti-tumour immune response *in vivo* via deposition as C3b and contribution to the amplification loop of the alternative pathway. Whilst this may contribute to anti-tumour immune responses, either through C-mediated lysis or opsonisation, C3b may alternatively be stimulating cells and promoting tumour

progression as discussed in Section 1.12. The exact mechanisms influencing such effects remain unclear though numerous cellular signalling and proliferative effects have been associated with C3b / MAC deposition including increased resistance to C (Reiter, Ciobotariu and Fishelson 1992), intra-cellular protein phosphorylation (Donin et al. 2003) and enhanced proliferation (Longo et al. 2005). These observations, and the propensity of non-transformed fibroblast cells to secrete C3 (Katz et al. 1989) suggested that C3 synthesis/secretion is a possible characteristic of fibrosarcoma cells derived using this model. However, ELISA data examining tissue culture s/n found no detectable trace of C3, C3b or iC3b in s/n following overnight incubation with confluent K6 populations. Purified C3 of known concentration was used to provide a standard curve, with tissue culture s/n measured against the curve. A previous study reported secretion of C3 by malignant melanoma cells with 350ng detectable by ELISA following incubation of s/n with 10^6 cells over a 24 hour period (Jean et al. 1997). However, absorbance values acquired from s/n were lower than the 20ng/ml threshold of sensitivity for the assay, even when s/n was concentrated 10-fold prior to testing. These data indicate that negligible or no C3 is secreted by these cells *in vitro*.

RT-PCR data provided a measure of mRNA transcription and is an extremely sensitive method able to detect small amounts of mRNA present in a sample. However, the assay was unable to identify CD55, CD59 or C3 mRNA in K6 cells. The experiment was controlled using hepatocytes derived from a WT mouse, with significant amplification observed associated with each protein. This confirmed the specificity of the primers used and the efficacy of this technique for this purpose. The findings support previous data from flow cytometric, Western blotting and ELISA analysis of these cells and suggest that CD55, CD59 and C3 are neither synthesised nor secreted *in vitro*.

Experiments described in subsequent Chapters aim to investigate the respective roles of individual C components and regulators in tumour survival and proliferation. In order to ensure suitability of K6 as a model for this purpose, and to obtain reliable data, analysis of *in vivo* growth was deemed necessary. The *in vivo* growth of an appropriate tumour line must be of high

incidence and provide a consistent model with minimal variability between experiments. As shown in Figure 3.11, the number of K6 cells inoculated had a potent effect on tumour growth with a low (10^4) K6 dose exhibiting poor survival *in vivo*. Data derived from higher doses (10^5 and 10^6) were similar and exhibited 100% tumour take in WT mice. Growth of cells was recorded following inoculation and found to exhibit normal tumour growth patterns. However, following a dose of 10^4 K6 cells, poor tumour incidence was observed with only 50% of mice inoculated developing a tumour (n=8). This compares unfavourably with incidences observed following inoculation with 10^5 or 10^6 cells, each of which caused 100% tumour incidence. The growth curves for the higher doses were found to be comparable and showed no difference in growth or survival. This indicates a threshold above which tumour cells are able to escape immune surveillance and below which, immune responses are sufficient to clear tumour dose and prevent growth. Although the findings suggest a threshold, tumour dose was not shown to affect growth rate following tumour occurrence. In this way, initial tumour dose appears to affect incidence rather than progression of K6 tumours. A high rate of tumour incidence is important for further work in elucidating differences in tumour growth between groups, as such a K6 dose of 10^5 cells was chosen for further experiments using this model. This inoculum was selected so as to grow tumours in a high proportion of mice injected, whilst not inoculating such a high burden as to 'over-burden' the host immune system which may potentially mask differences in tumour growth between different groups.

The K6 cell fibrosarcoma line appears well adapted for *in vitro* growth and provides an effective model allowing investigation of the effects of C proteins on tumour growth *in vivo*. Interestingly, these cells were shown not to express the important CReg CD55 and CD59 and also shown not to secrete the central C component C3 *in vitro*.

4. The effect of complement deficiencies on *in vivo* growth of the fibrosarcoma line K6

4.1. Introduction

The aims of the experiments described in this Chapter were to investigate the relative contributions of various C proteins to tumour growth *in vivo*. An established fibrosarcoma line, characterised in Chapter 3, was inoculated into groups of mice lacking C1q, C3, C5 or lacking both the CRegs CD55 and CD59. Each experiment was controlled using age-matched WT mice and tumour growth, C3b/iC3b and C9 deposition and $CD4^+$ infiltrate was analysed and compared between groups. As discussed in Chapter 1, protection from C is an important characteristic of all tumour cells. There is also evidence to support a role for C activation and deposition in tumour survival and proliferation. Knock-out mice deficient in C1q, C3 and lacking CRegs CD55 and CD59 were available in house providing *in vivo* models for investigation of the relative roles played by these proteins in tumour survival and growth. In addition, a monoclonal antibody (mAb) specific for mouse C5 protein was used to neutralise this protein in serum *in vivo* allowing effects associated with C5 cleavage and downstream activation products to be investigated. BB5.1 is a murine IgG1 mAb raised through immunisation of $C5^{-/-}$ mice (Frei, Lambris and Stockinger 1987) and has been shown to block C5a formation and inhibit MAC deposition (Wang et al. 1995). Comparisons between test and WT groups were carried out in order to reveal differences in tumour growth and incidence, C activation and T-cell infiltration to be assessed providing important clues as to the contributions of C1q, C3, C5 and CReg to tumour progression *in vivo*.

The C1q protein is the initiator molecule of the classical activation pathway upon Ab binding. C3 is a crucial molecule central to all three activation pathways and responsible for a wide range of immunological effects. Cleavage of the C5 protein represents activation of the terminal pathway and may lead to MAC deposition and ultimately cell lysis while CD55 and CD59 do not contribute to C activation and are instead protective molecules expressed

by host cells to inhibit C and limit autologous attack. These proteins are discussed in Chapter 1 and their respective roles in the C pathways are represented in Figure 1.1.

4.2. Results

4.2.1. Male mice possess higher haemolytic activity than female mice

The C system provides a rapid and effective means of lysing target cells through activation of one or more C pathways. Non-nucleated cells are particularly prone to C-mediated lysis and are regularly used to assess serum C potency (Porcel et al. 1993). Considerable differences have been observed in the abilities of mouse serum to lyse sensitised erythrocytes. This variation may be attributed to differing haemolytic activity of serum according to mouse strain (Terry, Borsos and Rapp 1964), or gender (Holt et al. 2001), used in the assay. For this reason, initial investigation of haemolytic capabilities of serum from male and female C57/BL6 mice was performed. Serum from 3 male and 3 female C57/BL6 mice was collected and incubated with rabbit erythrocytes coated with a mouse anti-rabbit erythrocyte Ab and able to activate the classical pathway *in vitro*. Erythrocytes incubated with H_2O (100% lysis) and those not incubated with serum (0% lysis) provided controls against which percent lysis mediated by a range of serum dilutions was calculated. Haemoglobin released by cells was recorded and expressed as percent lysis in Figure 4.1. As shown, serum from male mice was able to lyse cells far more readily than that obtained from female mice. A commonly used method for comparing C haemolytic activity involved calculation of the serum concentration required to cause 50% lysis in a given assay, known as CH50. However, whilst serum from male mice caused lysis of 50% erythrocytes at a concentration of ~60% serum, serum from female mice failed to lyse 50% erythrocytes and caused lysis of only 20% erythrocytes even with neat serum. Whilst a quantitative difference is therefore difficult to assess, a clear difference exists whereby serum from male mice is significantly more lytic than that from female mice. The observed disparity means that any

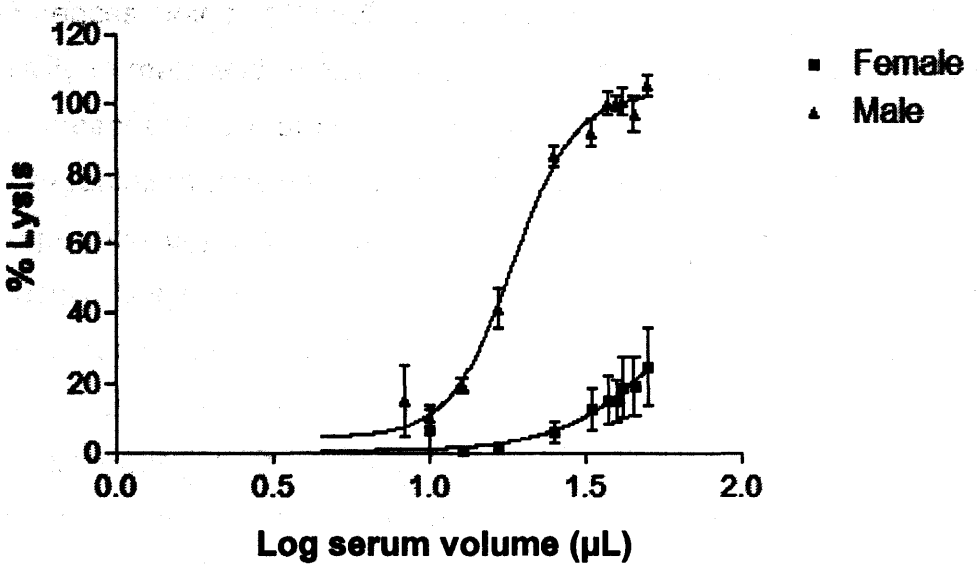


Figure 4.1: Haemolytic capabilities of male and female WT mice
Rabbit erythrocytes were sensitised using a mouse anti-rabbit erythrocyte pAb and incubated with serum from 3 male and female WT mice. Haemoglobin release following classical pathway activation was measured and expressed relative to a 100% control lysed with H_2O.

differences noted in tumour growth may arise as a result of differential C activity in male and female mice and make accurate conclusions concerning the impact of C on tumour growth difficult. For this reason, male mice would be expected to provide a better opportunity to elucidate differences in tumour progression dependent on C effects. Both males and females possess an effective C system *in* vivo, however, male mice were used in the majority of experiments described in this thesis. In the following experiments, WT mice and those deficient in specific proteins of interest were injected simultaneously with fibrosarcoma cells. Differences in tumour incidence, growth and survival as a result of C deficiencies were monitored.

4.2.2. The effect of C1q deficiency on K6 fibrosarcoma growth

Mice deficient in C1q were used to investigate the effects of activation of the classical C pathway on tumour growth *in vivo*. These mice were previously generated by targeted deletion of the C1q gene (Botto 1998). $C1q^{-/-}$ mice are unable to activate C in response to antibody binding to tumour cells and as such, all C-mediated effects must be elicited via the alternative or MBL pathways. By abrogating one potent pathway of C attack, it would seem likely that tumours would be subjected to a sub-optimal C response and therefore be less susceptible to CDC. In addition to direct C effects (e.g. killing, anaphylatoxin release), various other mechanisms are associated with C1q binding. These are discussed in detail in Chapter one and include opsonisation, cellular cytotoxicity and clearance of apoptotic cells. Mechanisms associated with C1q binding would be expected to be inhibited in $C1q^{-/-}$ and may impact on tumour progression in various ways. However the complexity and variety of such mechanisms suggest that a multitude of factors could contribute to any observed effects. Figure 4.2 shows growth rates as observed in 8 wild type and $C1q^{-/-}$ mice following sub-cutaneous injection of $1x10^5$ K6 cells. All mice developed a tumour with the majority arising between 4-8 days after inoculation. In each case, the tumour proliferated with similar growth kinetics (i.e. comparable slopes to the curves) and no clear differences between the groups were observed. However, tumours appeared to arise

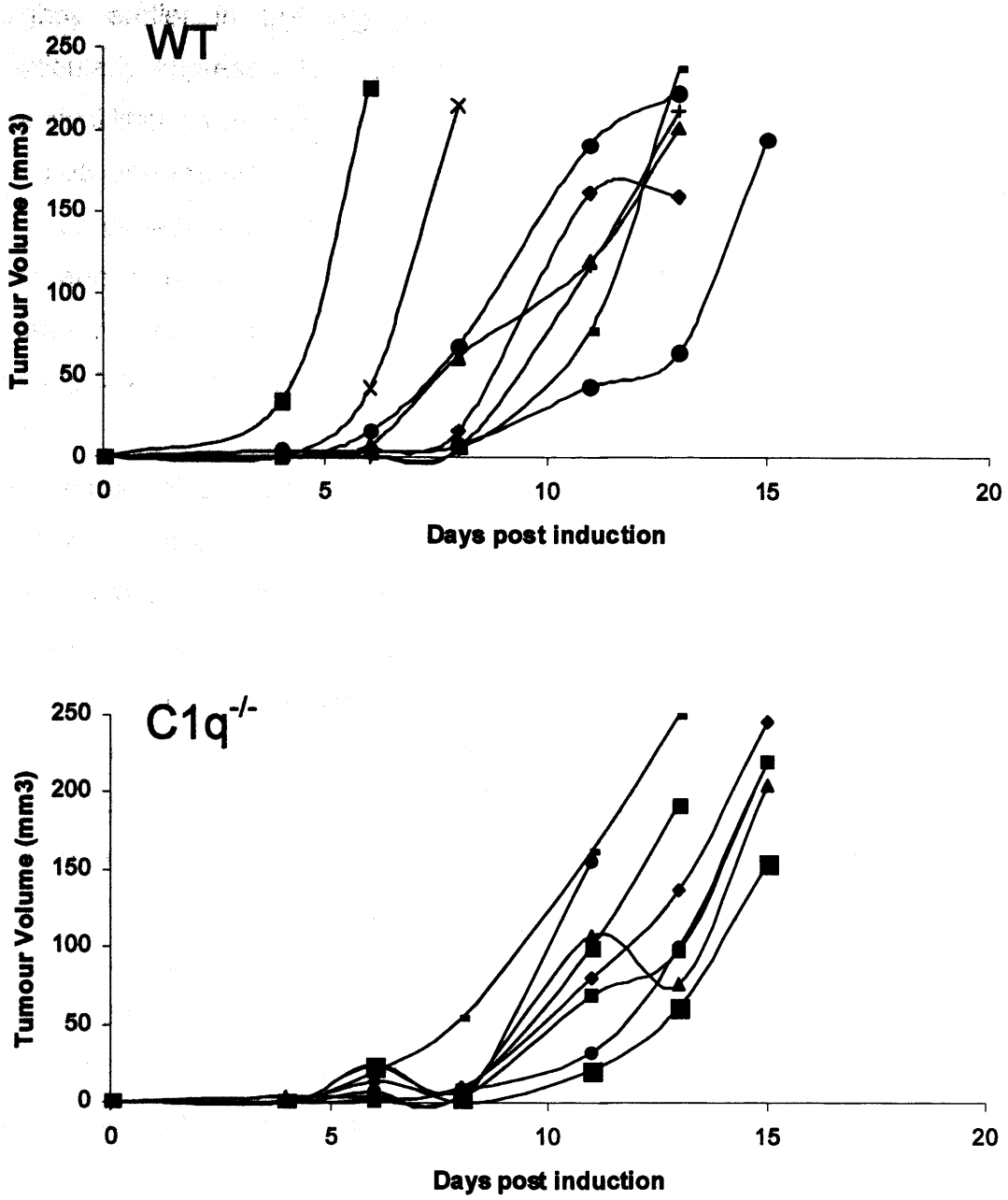


Figure 4.2: Growth of K6 fibrosarcoma line in WT and C1q$^{-/-}$ male mice
Eight WT and C1q-/- mice were inoculated with 10^5 K6 cells. Mice were injected on day 1 with 100μL tumour cells on the flank and monitored regularly with tumour sizes measured. Each line is representative of one individual mouse. Mice were sacrificed when tumour reached 200mm^3. Data shown (n=8) are representative of 3 separate experiments.

slightly earlier in the WT group with 2 tumours in particular growing particularly aggressively. Although every care was taken to use genetically identical littermates and identical tumour cell doses, differences within a group i.e. between identically treated littermate control mice is inevitable. Although tumour growth showed consistency within groups, it is difficult to analyse differences between groups in terms of tumour growth rate. Instead, alternative parameters such as tumour incidence or survival, are more appropriate to analyse differences and assess statistical significance.

The impact of C1q deficiency on tumour growth was further analysed using pooled data from 3 (n=8) separate and identical experiments (n=24). In each case, differences between the groups were analysed using the log-rank test. Figure 4.3 shows tumour incidence in WT and $C1q^{-/-}$ mice. The incidence graph plots percent of mice bearing a visible tumour while the survival graph shows number of mice tumour free, or bearing a tumour smaller than the threshold for sacrifice. These graphs demonstrate any difference in time taken to form a tumour of $200mm^3$ following cell inoculation. Neither graph reveals a discernable visual difference between K6 incidence or survival of WT and $C1q^{-/-}$ mice. This observation is reflected by the non-statistically significant p values (0.178 and 0.715) obtained from these data via the log-rank statistical test. Based on these data, K6 appears to grow in WT and $C1q^{-/-}$ male mice with similar incidence, survival and growth rates.

4.2.3. The effect of C3 deficiency on K6 fibrosarcoma growth in mice

Mice deficient in the central C component C3 have been generated previously by targeted disruption of the C3 gene (Pekna et al. 1998). These mice were used to investigate the large scale effect of C activation on tumour progression *in vivo*. WT mice possess a fully functional C system and activation and deposition of C components has been demonstrated (Niculescu et al. 1992, Magyarlaki et al. 1996). Conversely, only the initial steps prior to C3 cleavage are possible in the $C3^{-/-}$ mice. Therefore no alternative pathway activation is possible. C1q or MBL can bind targets, allowing cleavage of C4

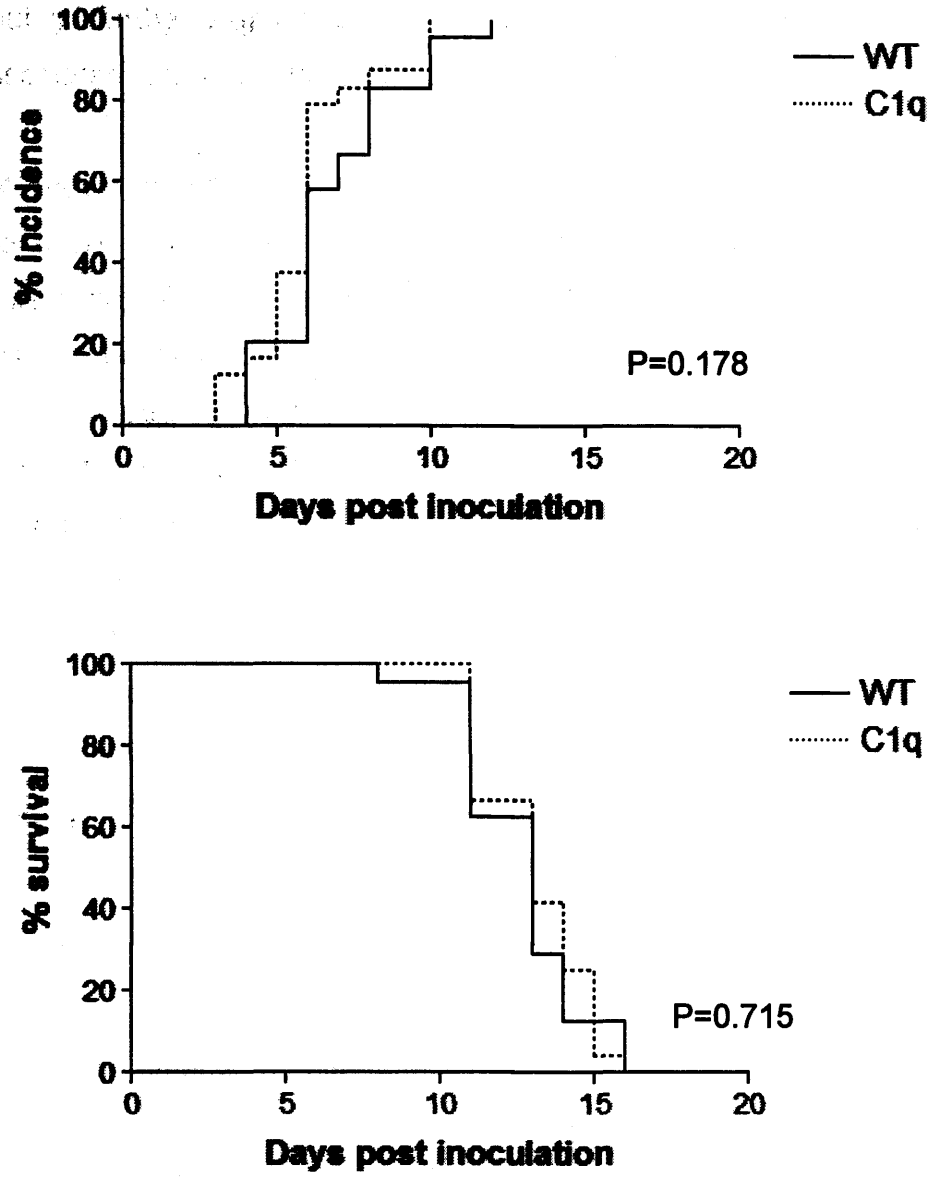


Figure 4.3: K6 incidence and survival in WT and C1q$^{-/-}$ male mice
Pooled data from WT male (n=24) and C1q$^{-/-}$ male (n=24) .Mice were inoculated with 10^5 K6 cells, sub-cutaneously into the left flank. K6 incidence occurs when a visible tumour is observed, while survival events are marked when tumour reached pre-determined volume (200mm^3). Differences between the two groups were analysed using the log-rank test. The effects were found not to be statistically significant ($p>0.05$)

and C2 but no further steps in any C pathway, nor any of the downstream effects associated with C are possible.

Figure 4.4 shows growth of K6 tumours following inoculation into 8 WT and $C3^{-/-}$ male mice. These data are pooled from three separately controlled experiments (n=8). As with the previous experiment (Figure 4.2), no visual differences can be seen between the groups. Reduced regulation of tumour growth (by C) would allow tumours to arise earlier and/or show a faster proliferation rate visualised through a steeper curve. As previously, these data are more appropriately assessed by the use of incidence and survival curves and statistically analysed using the log-rank test. Figure 4.5 shows no differences in time taken for tumours to arise between the two groups, there was also no difference observed in survival. The p values of 0.442 and 0.736 respectively reflect this and suggest that C deposition and associated effects are not important either for the control or development of K6 tumours.

4.2.4. The effect of C5 neutralisation on K6 fibrosarcoma growth in mice

A blocking mAb against mouse C5 is available and was harvested and purified in house. Hybridoma cells were cultured as described in Chapter 2, supernatant was collected and antibody purified using Protein G affinity chromatography. Figure 4.6 shows reduced and non-reduced samples of antibody stained with Coomassie blue. The antibody sample is pure and non-aggregated with the native protein forming a single band at ~150kDa. The reduced portion shows a heavy chain band at ~50kDa and the light chain at ~25kDa. These weights are consistent with known Ab structures. An IgG Ab consists of two heavy and two light chains linked by disulphide bonds and forming a native protein of ~150kDa (Edelman, 1969). Mice administrated with an appropriate dose of this Ab are rendered 'C5-neutralised' and provide a useful model for observing the impact of C5 on tumour growth.

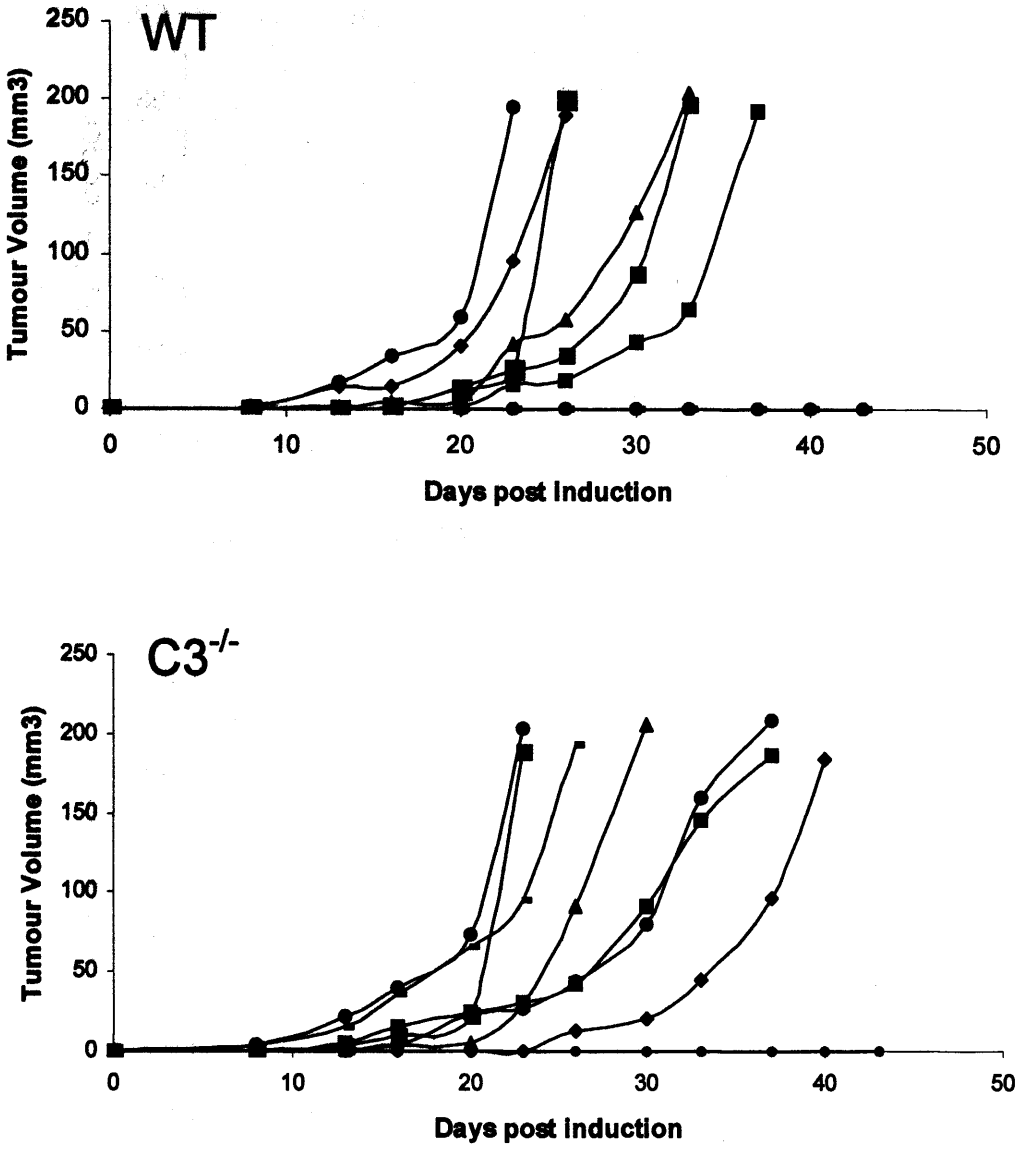


Figure 4.4: Growth of K6 fibrosarcoma line in WT and C3-/- male mice
Eight WT and C3-/- mice were inoculated with 10^5 K6 cells. Mice were injected on day 1 with 100μL tumour cells on the flank and monitored regularly with tumour sizes measured. Each line is representative of one individual mouse. Mice were sacrificed when tumour reached 200mm^3. Data shown (n=8) are representative of 3 separate experiments.

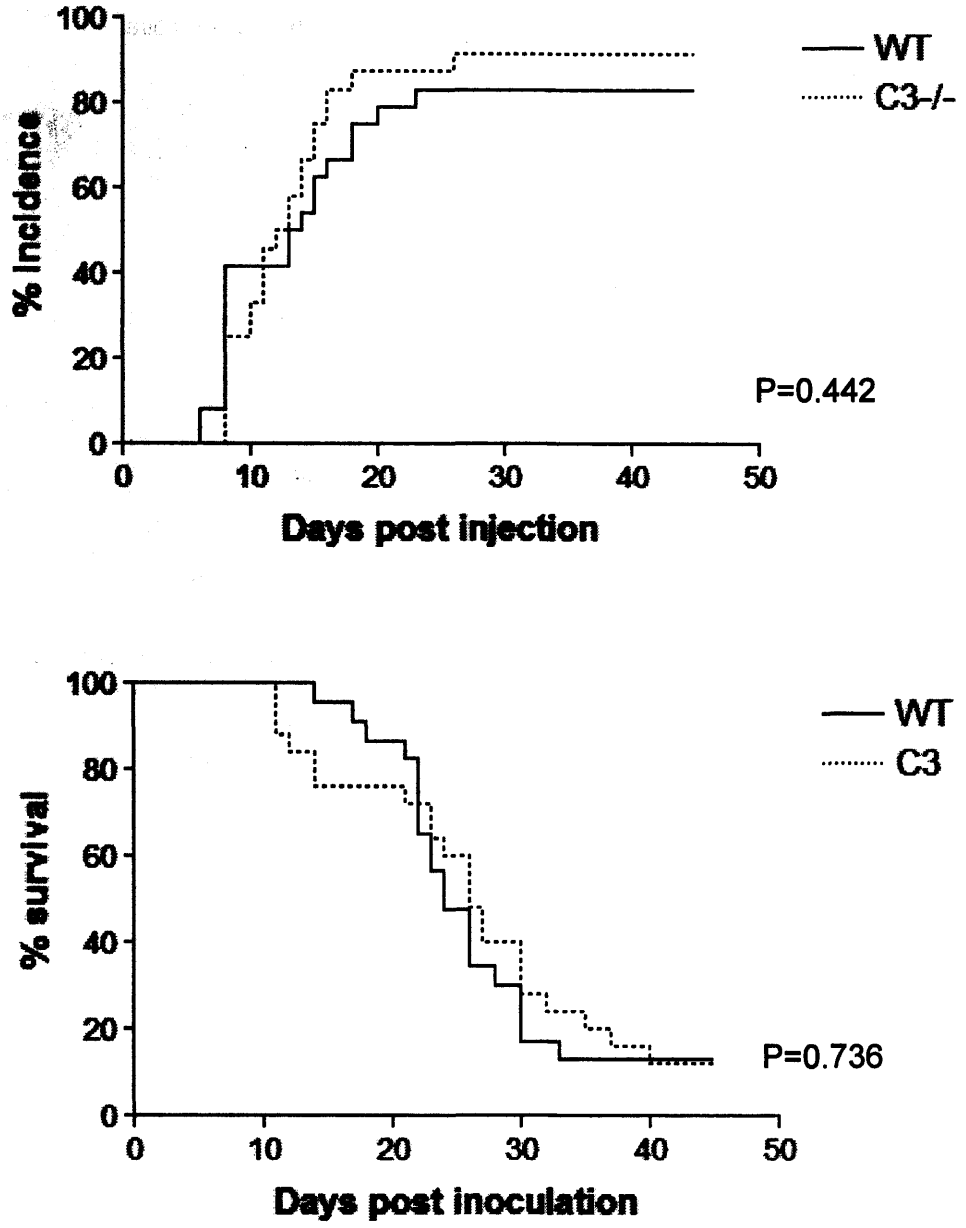


Figure 4.5: K6 incidence and survival in WT and C3-/- male mice
Pooled data from WT male (n=24) and C3-/- male (n=24) .Mice were inoculated with 10^5 K6 cells, sub-cutaneously into the left flank. K6 incidence occurs when a visible tumour is observed, while survival events are marked when tumour reached pre-determined volume (200mm^3). Differences between the two groups was analysed using the log-rank test. The effects were found not to be statistically significant ($p>0.05$)

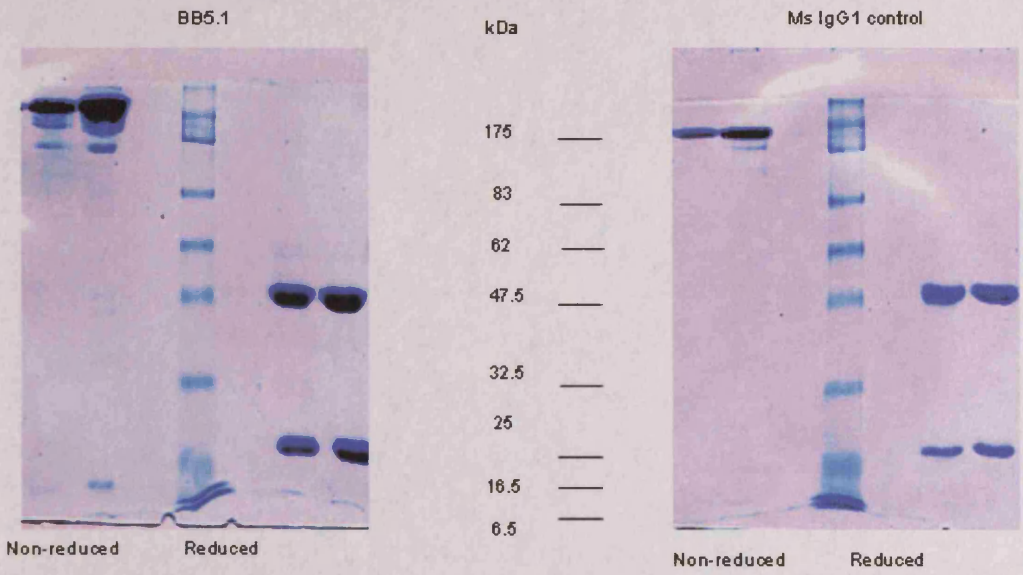


Figure 4.6: Coomassie stained reduced and non-reduced antibody samples
7.5% gel electrophoresis (SDS-PAGE) stained with Coomassie blue. BB5.1 and isotype control antibody were loaded at 2.5µg (left) and 5µg (right) in native (non-reduced) and reduced (β-mercaptoethanol treated) form. NEB broad range pre-stained protein markers of known size were loaded simultaneously (middle lane) to allow mass estimation.

4.2.4.1. Neutralisation of C5 by BB5.1 administration

In order to neutralise C5 over the course of tumour growth, dose of BB5.1 was first titrated to ensure circulating C5 was neutralised then further doses of BB5.1 were administered to remove newly synthesised C5. Since C5 is required for activation of the terminal pathway and the lytic effector MAC, assessment of haemolytic capability was deemed an appropriate way to determine whether C5 was available or had been de-activated. Figure 4.7 shows *in vitro* blocking of C5 and subsequent abrogation of haemolytic capability. Serum was incubated with varying amounts of BB5.1 and shown to possess lower haemolytic activity dependent on dose of mAb administered. Concentration of BB5.1 was plotted against percent inhibition following incubation. The dose required to neutralise C5 activity over the course of an experiment depends on numerous factors including C5 plasma concentration, weight of the animals, rate of removal from the bloodstream and rate of resynthesis. Based on the *in vitro* experiment illustrated in Figure 4.7, a dose of 2mg BB5.1 anti-mouse C5 was chosen to test C inhibition *in vivo*. BB5.1 was injected intra-peritoneally, blood was sampled at regular intervals and serum harvested. A classical pathway haemolysis assay was used to determine the lytic capabilities of serum at different timepoints post mAb dose. Figure 4.8 shows that haemolytic capability is lost until day 6 in male mice and day 11 in female mice. Haemolytic activity slowly returned as *de novo* synthesis replaced C5 and remaining BB5.1 antibody was either bound or removed from circulation in these animals. The differences observed between sexes are likely to be due to male mice being larger than female counterparts at adulthood – thus males possess a greater total blood volume and therefore total C5 amounts in serum vary between sexes. However, the amount of C5 present per ml blood may also be gender dependent. A dose of 1mg BB5.1 per mouse, administered at 4 day intervals was chosen to ensure complete neutralisation of C5 throughout the period of tumour growth, this dose was consistent with that used by other groups (Liu et al. 2005). In order that C5 was neutralised prior to tumour inoculation, the initial BB5.1 dose was administered 1 day prior to K6 inoculation.

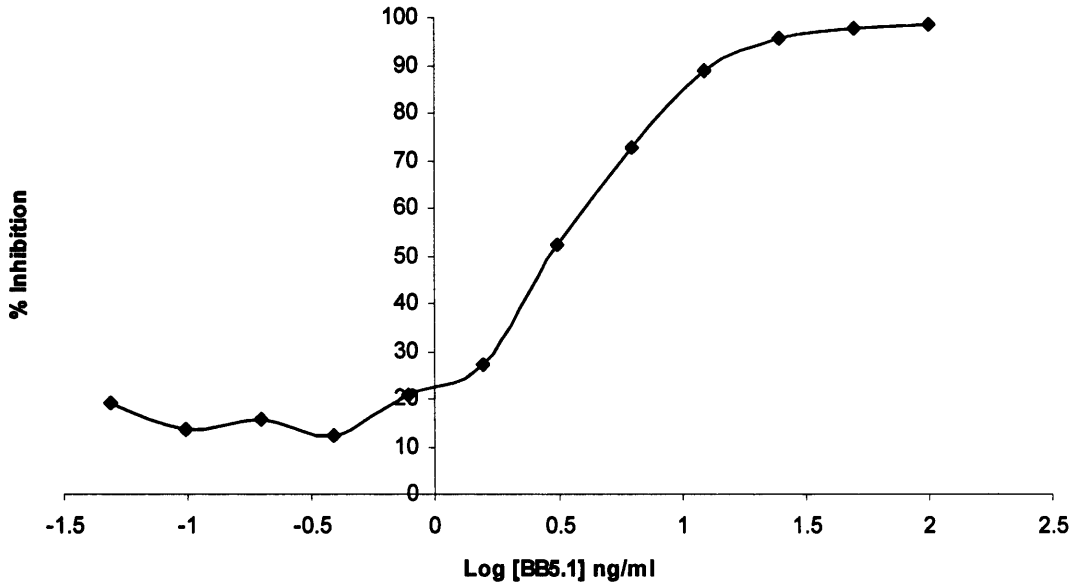


Figure 4.7: *In vitro* inhibition of complement by C5 depletion
Rabbit erythrocytes were sensitised by incubation with a mouse anti-rabbit erythrocyte pAb and treated with normal mouse serum pre-incubated with various titres of BB5.1 to deplete C5. Haemoglobin release was measured and expressed relative to 100% control erythrocytes lysed using H_2O.

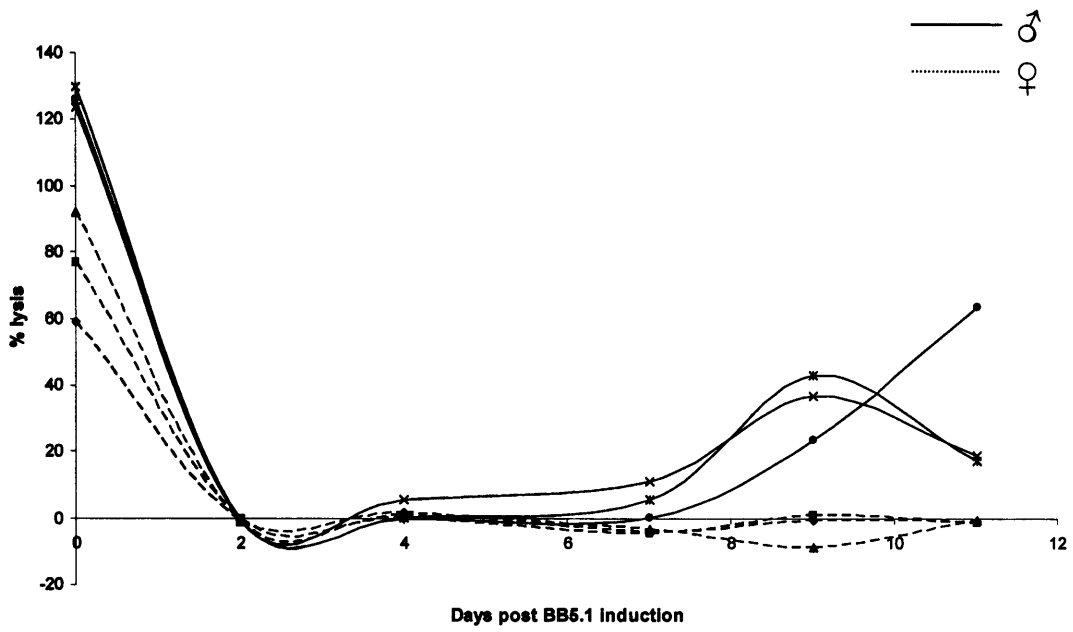


Figure 4.8: *in vivo* inhibition of complement in male and female WT mice
Three male and female WT mice were treated with a 2mg BB5.1 dose. Serum haemolytic capabilities were assessed via classical pathway activation on rabbit erythrocytes sensitised using a mouse anti-rabbit erythrocyte pAb. Lysis was measured relative to 100% control erythrocytes lysed in H_2O. Bold lines represent individual male wild type mice, dashed lines represent female mice.

4.2.4.2. K6 tumour growth in C5 neutralised male mice

In order to investigate the contribution of C5 to tumour growth in this model, mice were administered with a neutralising Ab and controlled with age-matched untreated mice. Due to differences observed in Figure 4.1, male and female groups were analysed separately and compared only with same gender groups. Figure 4.9 shows tumour growth in male and female mice treated with BB5.1 and the control group without Ab (n=6). Initial observation suggests that tumours grew more slowly in male mice following BB5.1 administration. The C5-neutralised male mice experienced a slower rate of K6 tumour growth, i.e. a less steep growth curve, as compared to non-treated control mice. Additionally, these mice appeared to survive for longer. As with previous experiments, tumour growth curves do not provide a good parameter for assessing statistical significance. This effect was not mirrored in the female group, perhaps due to the lower haemolytic activity and therefore less pronounced effects following C activation. Interestingly, one male and one female mouse (from groups of 6) treated with BB5.1 failed to develop a tumour compared to 100% tumour take in the non-treated mice. Figure 4.10 illustrates increased survival in mice treated with BB5.1 and revealed a strong protective effect found to be statistically significant ($p=0.005$) upon analysis using the log-rank test. Therefore, neutralising C5 in male mice protects against K6 tumour growth and suggests a role for C5 in promoting tumour progression in untreated hosts.

Figure 4.10 also shows survival data from the female groups and supports the earlier observation from Figure 4.9 that significant protection is not observed in these mice. Although one mouse did not develop a tumour, the initial trend in females was for Ab-treated mice to be sacrificed at earlier timepoints as tumours reached their maximum size more rapidly. This indicates a faster growth of tumours in these mice. However, the area between the survival curves is considerably less than that observed in male groups and a p-value of 0.938 confirms there is no statistically significant difference. Figure 4.10 also demonstrates incidence curves derived from the same data. K6

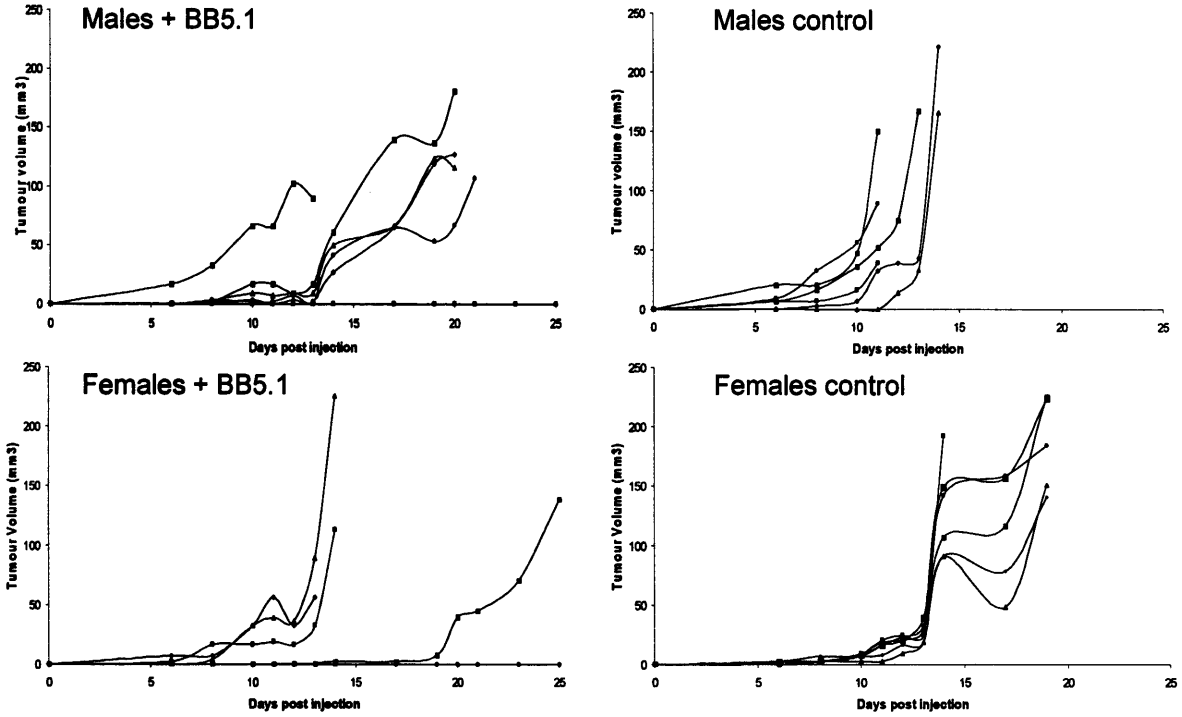


Figure 4.9: Growth of K6 fibrosarcoma line in WT and C5-depleted mice
K6 fibrosarcoma cells were inoculated into C5-depleted (2 mg dose I.P. on day -1, day 6, day 13 and day 21) and non-treated male and female mice. Each line represents one individual mouse, six mice per group. Mice were sacrificed when tumour size exceeds 150mm^3.

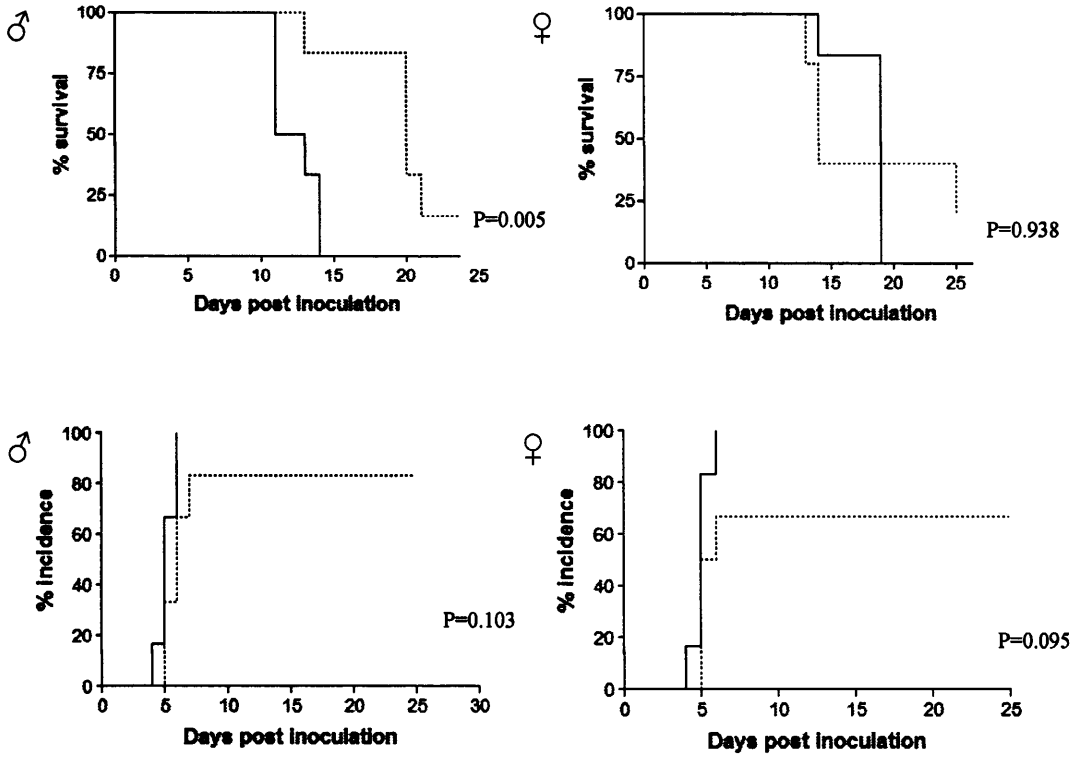


Figure 4.10: K6 incidence and survival in WT and C5-depleted mice
K6 fibrosarcoma growth in C5-depleted (2 mg dose I.P. on day -1, day 6, day 13 and day 21) and non-treated male and female mice. Bold lines represent WT mice, dashed represent C5-depleted. K6 incidence occurs when a visible tumour is observed, while survival events are marked when tumour reached pre-determined volume ($200mm^3$).

occurrence is plotted for male and female mice, both in WT and C5-neutralised groups. These data lend support to the earlier findings and suggest a possible protective trend following C5 neutralisation, however, the differences between groups are less evident and this is reflected in the higher and non-statistically significant p-values of 0.103 and 0.095 respectively.

In order to ensure the validity of these results, three experimental parameters required addressing. Firstly, larger group sizes were necessary to ensure experimental significance. Secondly, an appropriate isotype control Ab (mouse IgG1) was required to control for non-specific binding of BB5.1 to cellular or serum proteins and Ab aggregation effects which may affect tumour growth non-specific to effects of C5 neutralisation. Use of a control mouse IgG1 Ab at the same concentration would prove that the specific and direct effects of C5 neutralisation were responsible for the protective effect. Thirdly, post-experimental testing of the antibody sample found high titres of endotoxin contamination (>30EU/ml). Lipopolysaccharide (LPS) detected within the Ab samples can have large scale effects on numerous immune functions, most notably activation of phagocytic cells (reviewed by Heine et al. 2001) and may significantly distort the immune response to injected tumour cells used in this model. Although an isotype control antibody prepared via these same methods would likely contain comparable levels of endotoxin contamination and therefore control for the specificity of the anti-C5 effect, the immunostimulatory and inflammatory properties of LPS would create a non-physiological environment for tumour growth, casting doubt upon the validity of the findings.

In order to remove endotoxin from the antibody preparations, a polymixin B preparation ('Detoxigel' supplied in columns by Pierce Biotechnology) was used as described in Chapter 2. Despite attempts to remove endotoxin contamination from antibody preparations, levels of endotoxin remained high (>25EU/ml). In order to generate clean preparations for reliable data, new BB5.1 and a mouse IgG1 anti-human C4 antibody (chosen as a control and demonstrated not to cross-react with murine C4) were purified. This involved using endotoxin free reagents and rigorous steps to minimise contamination

and is described in detail in Chapter 2. Upon testing, endotoxin concentrations of 0.38 EU/ml and 0.40 EU/ml respectively were found. These readings were provided by the endotoxin screening service (Department of Medical Microbiology, Heath Park, Cardiff University). Standard endotoxin limits for commonly used animal models in preclinical research suggest that for a typical mouse, endotoxin dose should be below 1.5EU/ml as defined by the United States Food and Drug Administration (USFDA) (Malyala and Singh 2008).

The isotype control antibody was obtained from Dr Claire Harris, Cardiff University and is specific for human C4. To ensure, that this antibody does not cross-react with mouse C4, a haemolytic test was performed. Figure 4.11 shows that BB5.1 and not the isotype control Ab was able to inhibit C-mediated haemolysis and that the control Ab has no effect on haemolytic capability of mouse serum. Whilst this data shows that the mAb does not affect the haemolytic capacity of mouse serum, it does not prove that the anti-human C4 mAb is not binding to mouse C4. In order to test this, a Western blot was performed and is shown in Figure 4.12. A band for the C4 protein is detected in lanes loaded with human serum, but not with mouse serum. Other bands shown are also present in the control blot, treated with secondary Ab only and represent IgM and IgG heavy chains non specifically bound by the rabbit anti-rat IgG secondary. Together these data show that the mouse anti-human C4 mAb does not cross react with mouse C4 and provides a suitable isotype control for the mouse anti-mouse C5 mAb.

Figure 4.13 shows tumour cell growth in male mice treated with endotoxin-low BB5.1 or anti-C4. In this experiment, a larger group size was used (n=12) although numbers were limited due to large antibody doses required and the available supply of Ab. Data shown in Figure 4.13 do not allow any differences between groups to be clearly discerned. However there was a trend for mice to be sacrificed later in the C5-neutralised group. Additionally, three of these mice remained tumour free compared to one in WT group. Figure 4.14 shows incidence and survival curves derived from these data. The survival curves clearly demonstrate a protective effect of C5 neutralisation

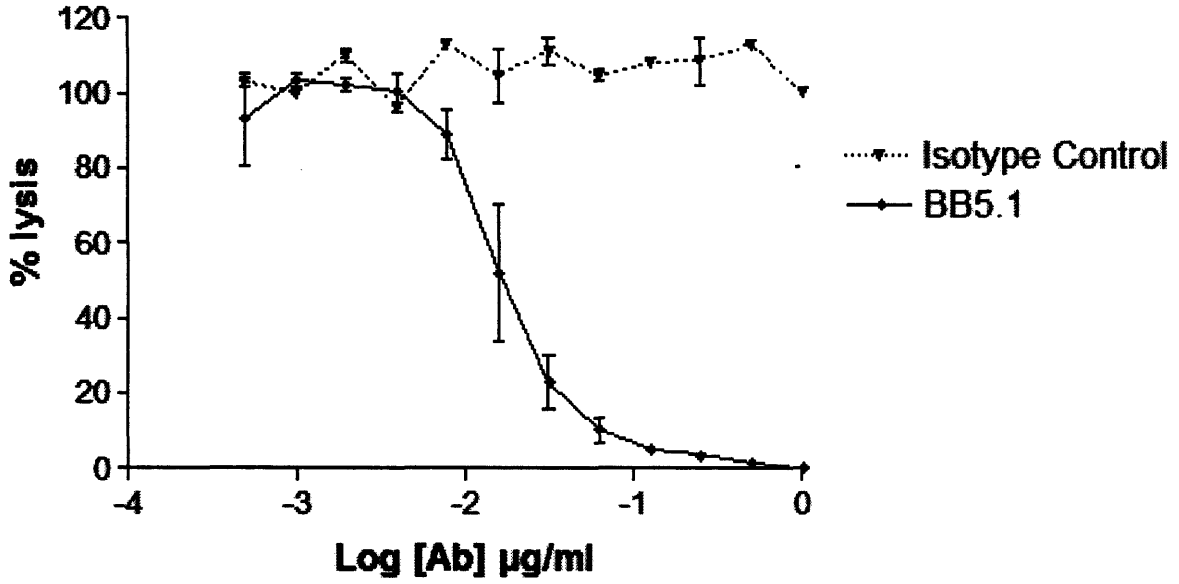


Figure 4.11: *in vitro* inhibition of complement by BB5.1
Rabbit erythrocytes were sensitised using 0.5% mouse anti-rabbit erythrocyte pAb and treated with normal mouse serum pre-incubated with various titres of BB5.1 or an isotype control Ab. Haemoglobin release was measured and expressed relative to 100% control erythrocytes lysed using H_2O. Serial dilutions assayed in triplicate.

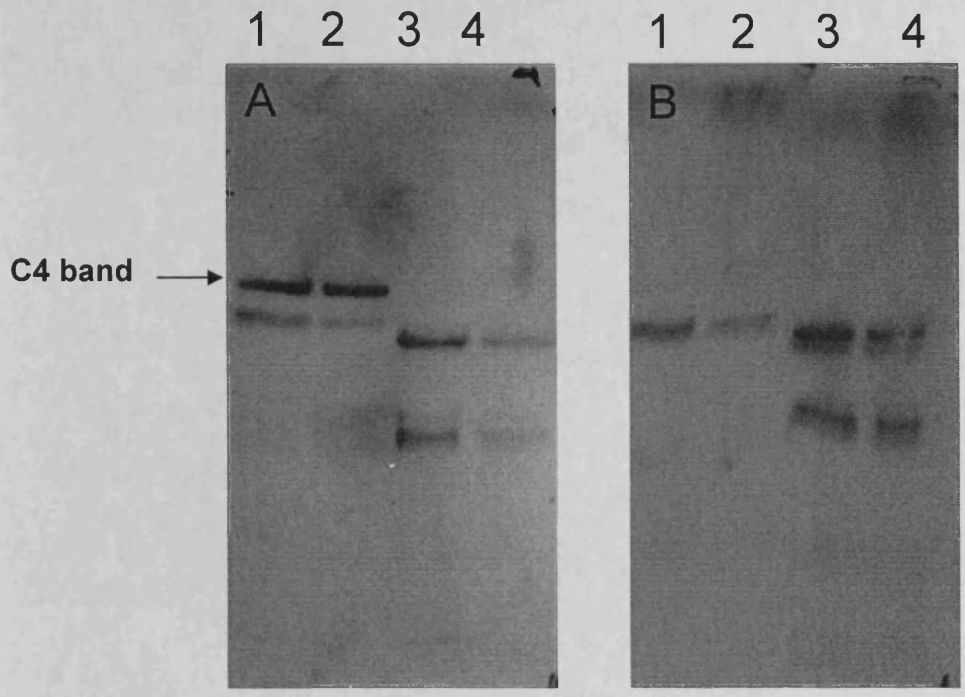


Figure 4.12: Western blot analysis of anti-C4 mAb binding to mouse / human serum
Lanes 1 and 2 were loaded with reduced normal human serum (160ng and 80ng C4 respectively based on normal serum level 0.6mg/ml). Lanes 3 and 4 contain reduced serum (160ng and 80ng C4 respectively) from a WT mouse. Panel A is stained with monoclonal mouse anti-human C4 at 10μg/ml, followed by 1:2000 HRPO-conjugated rabbit anti-mouse IgG. Panel B is stained with rabbit anti-mouse IgG only.

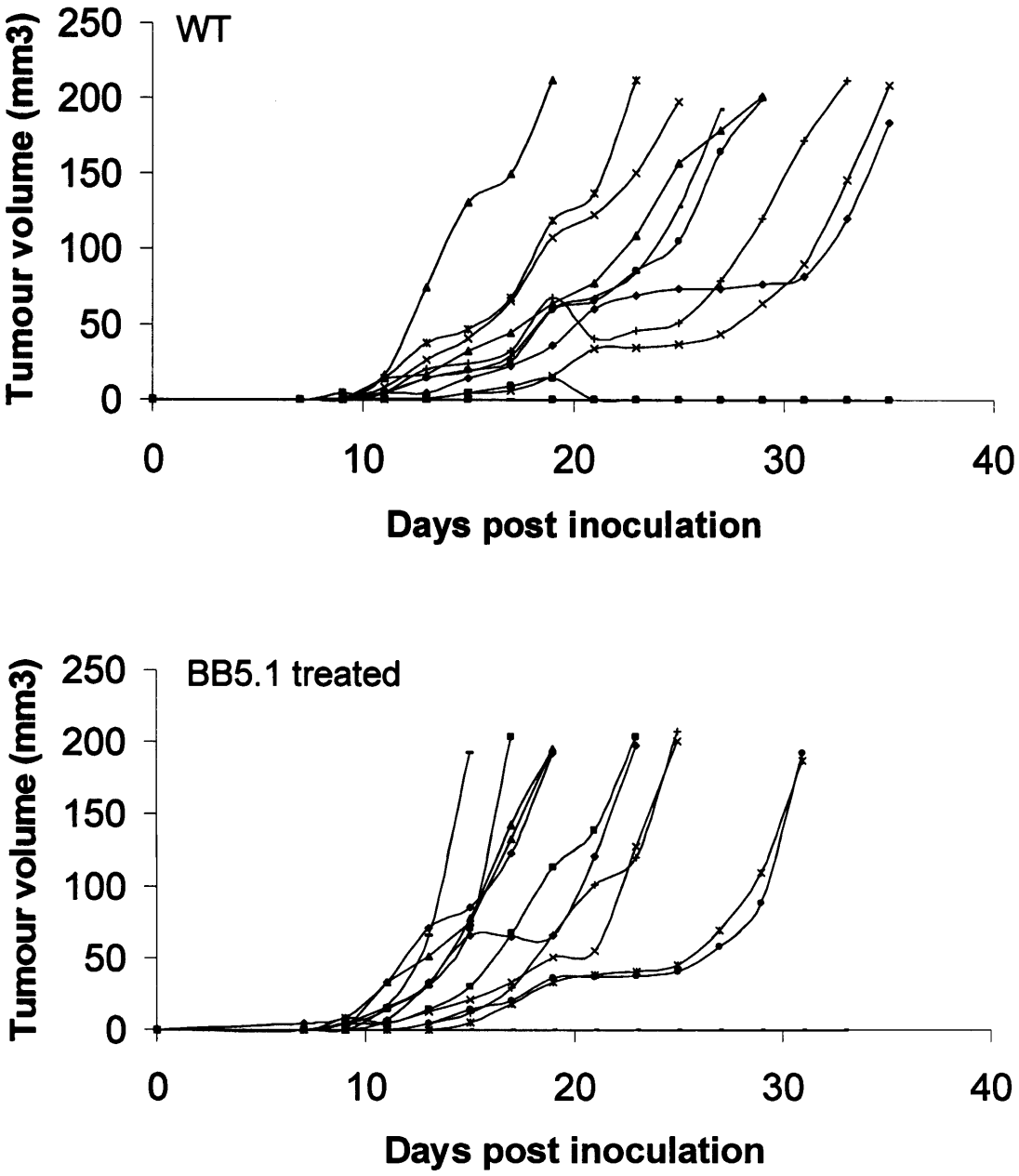


Figure 4.13: Growth of K6 fibrosarcoma line in WT and C5 neutralised male mice
WT and C5-neutralised mice were inoculated with 10^5 K6 cells. Mice were injected on day 1 with 100µL tumour cells on the flank and monitored regularly with tumour sizes measured. Each line is representative of one individual mouse. Mice were sacrificed when tumour reached 200mm^3. Each group consists of 12 mice. Each line is representative of one individual mouse.

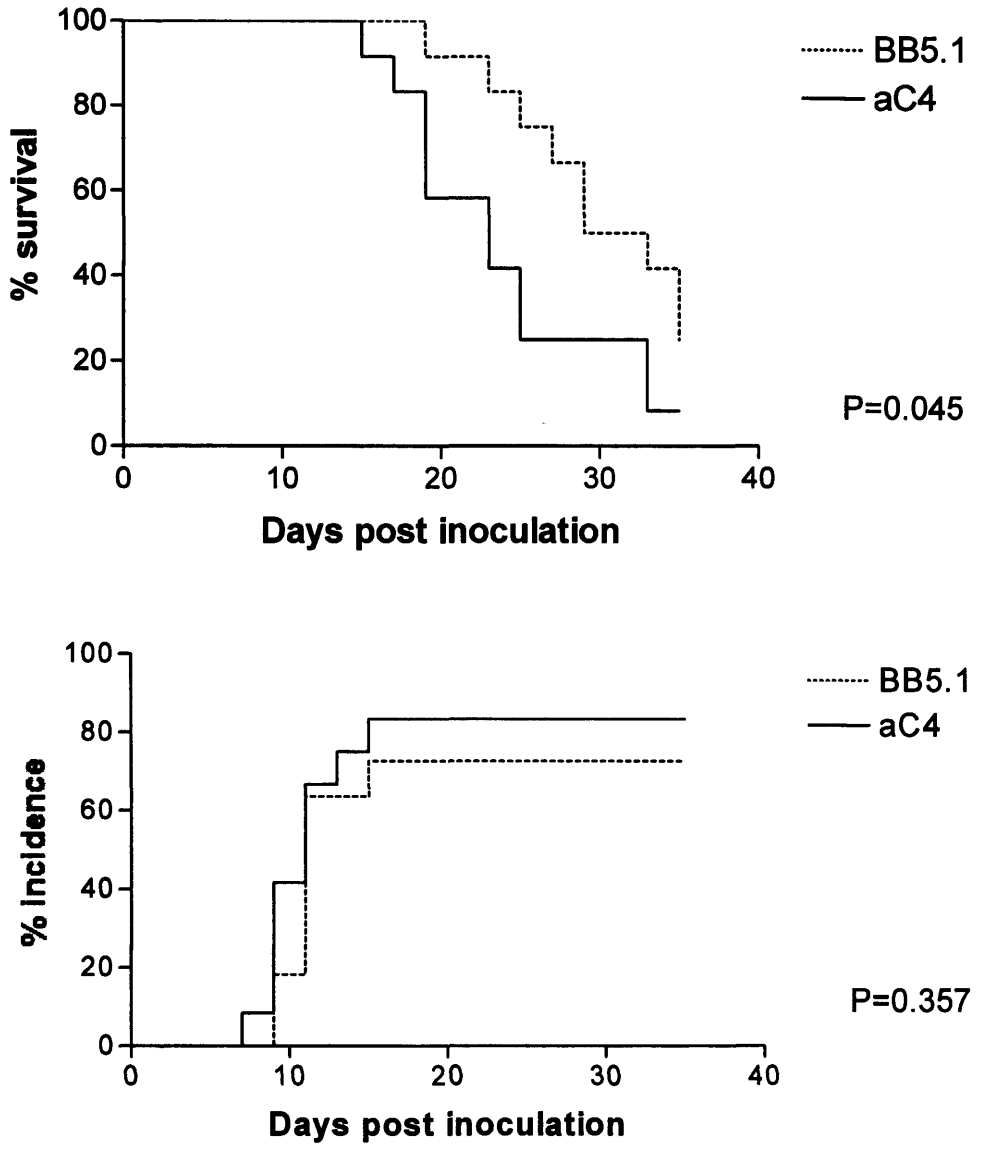


Figure 4.14: K6 survival and incidence in WT and C5-depleted male mice
K6 fibrosarcoma growth in C5-depleted (BB5.1) and isotype control treated male mice. Bold lines represent WT mice, dashed represent C5-depleted. Survival data is shown above, incidence data below. K6 incidence occurs when a visible tumour is observed, while survival events are marked when tumour reached pre-determined volume ($200mm^3$). Differences between WT and C5-depleted curves were analysed using the log rank statistical test.

compared to the control group with the effect found to be statistically significant (p=0.046). This supports the previous findings of retarded tumour growth in these animals. However, tumour incidence data also in Figure 4.14 do not show a significant difference between C5-neutralised and control groups. Collectively, these data show that C5 neutralisation retards, but does not completely suppress, growth of K6 tumours in male mice. It is therefore possible that C5 cleavage, causing C5a release and activation of the terminal pathway in WT host animals is important and contributes to driving tumour proliferation.

4.2.5. The effect of CD55 and CD59 deficiency on K6 fibrosarcoma growth

CD55 and CD59 are potent CReg involved in protection of numerous tumour cells types from CDC (Li et al. 2001, Yu et al. 1999). However, more recent reports have highlighted important immunological roles for these proteins un-related to CDC. Both proteins have been shown to possess down-modulatory functions on T-cells reviewed by (Longhi et al. 2006). Additionally, since host cells close to tumour cells lack these CReg, large scale C activation and inflammation may occur in the tumour environment. Mice of this genotype have previously been shown to be more susceptible to induced glomerulonephritis, attributable to CD55 deficiency and associated with increased C3b deposition and inflammation compared to WT controls (Bao et al. 2007). Exacerbation of the autoimmune disease myasthenia gravis is also reported in these mice, with both CReg contributing to protection in WT mice (Kaminski et al. 2006). Investigation of tumour growth in mice lacking these CReg may provide an insight into the innate and adaptive immune mechanisms affecting tumour growth in this model.

$CD55^{-/-}.CD59^{-/-}$ mice possess all the components for a normal C response. However since all host tissues lack two key CReg, impaired C regulation would be expected, and may lead to increased consumption of C components and a lower lytic capacity. Data described in Chapter 3 showed that K6 cells

do not express CD55 or CD59 *in vitro,* however, these cell lines possess the genes encoding both proteins and may up-regulate their expression in response to C deposition or cytokine stimuli (Spiller et al. 2000). Figure 4.15 shows that mice deficient in CD55 and CD59 possess equivalent haemolytic activity to WT mice. This suggests that circulating C components are not consumed in $CD55^{-/-}.CD59^{-/-}$ animals and that activation of C pathways in these or WT hosts will cause similar initial effects. However, following activation, impaired inhibition on $CD55^{-/-}.CD59^{-/-}$ host tissues may result in enhanced deposition and inflammation around activating surfaces i.e. tumour cells. Figure 4.16 shows tumour cell growth in 8 male wild types and 8 male $CD55^{-/-}.CD59^{-/-}$ mice. No statistically significant difference between the groups is evident from the growth curves. However Figure 4.17 shows incidence and survival curves from pooled data and identified a positive trend correlating absence of CD55 and CD59 in hosts with earlier incidence of K6 tumours and similarly earlier termination of these animals. However, the effects were not statistically significant ($p=0.162$, $p=0.105$) although higher group sizes may be needed to further examine this trend and to reach a more definitive conclusion.

4.2.6. C3b/iC3b deposition on K6 tumour tissue

Deposition of C3 activation fragments on K6 tumour sections taken from WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice can be analysed using immunohistochemical techniques. The mAb used recognises native C3, the active deposited fragment C3b and the associated inactivation product iC3b (Mastellos et al. 2004). Deposition of C3b provides a valuable marker of C activation and targeting to tumour cell surfaces. Figure 4.18 shows a representative slide from a K6 tumour section grown in each of WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ genotypes. Each picture is representative of 8 sample image slices taken from 6 separate tumours inoculated into each mouse strain. C3b/iC3b deposition was observed on each tumour section analysed except those inoculated into $C3^{-/-}$ animals. This indicates that K6 tumours are stimulating one or more pathways of C activation. In the case of

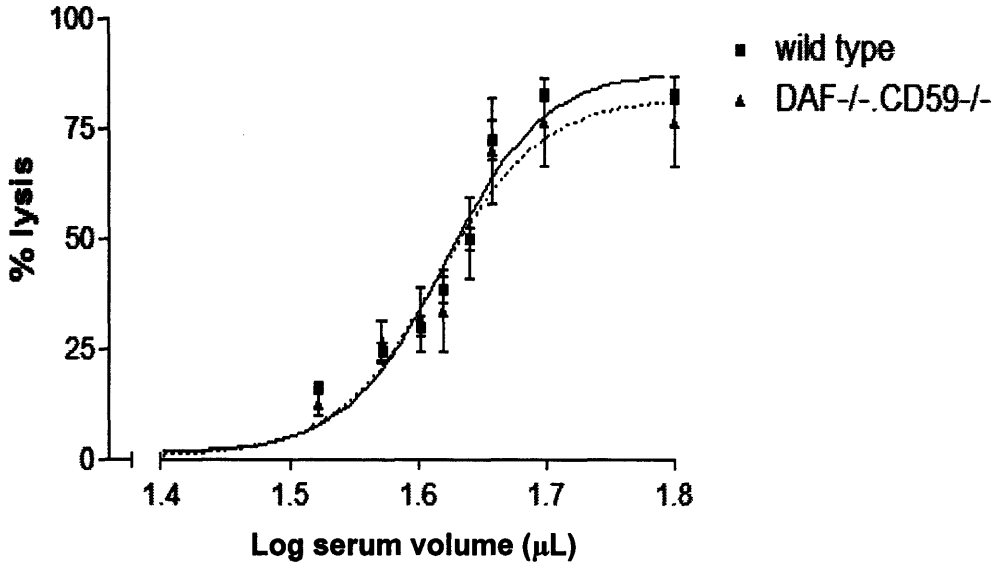


Figure 4.15: Haemolytic capabilities of male WT and CD55$^{-/-}$.CD59$^{-/-}$ mice
Rabbit erythrocytes were sensitised with mouse anti-rabbit erythrocyte pAb and incubated with serum from 3 male WT and CD55$^{-/-}$.CD59$^{-/-}$ mice. Haemoglobin release was measured and expressed relative to a 100% control lysed with H_2O.

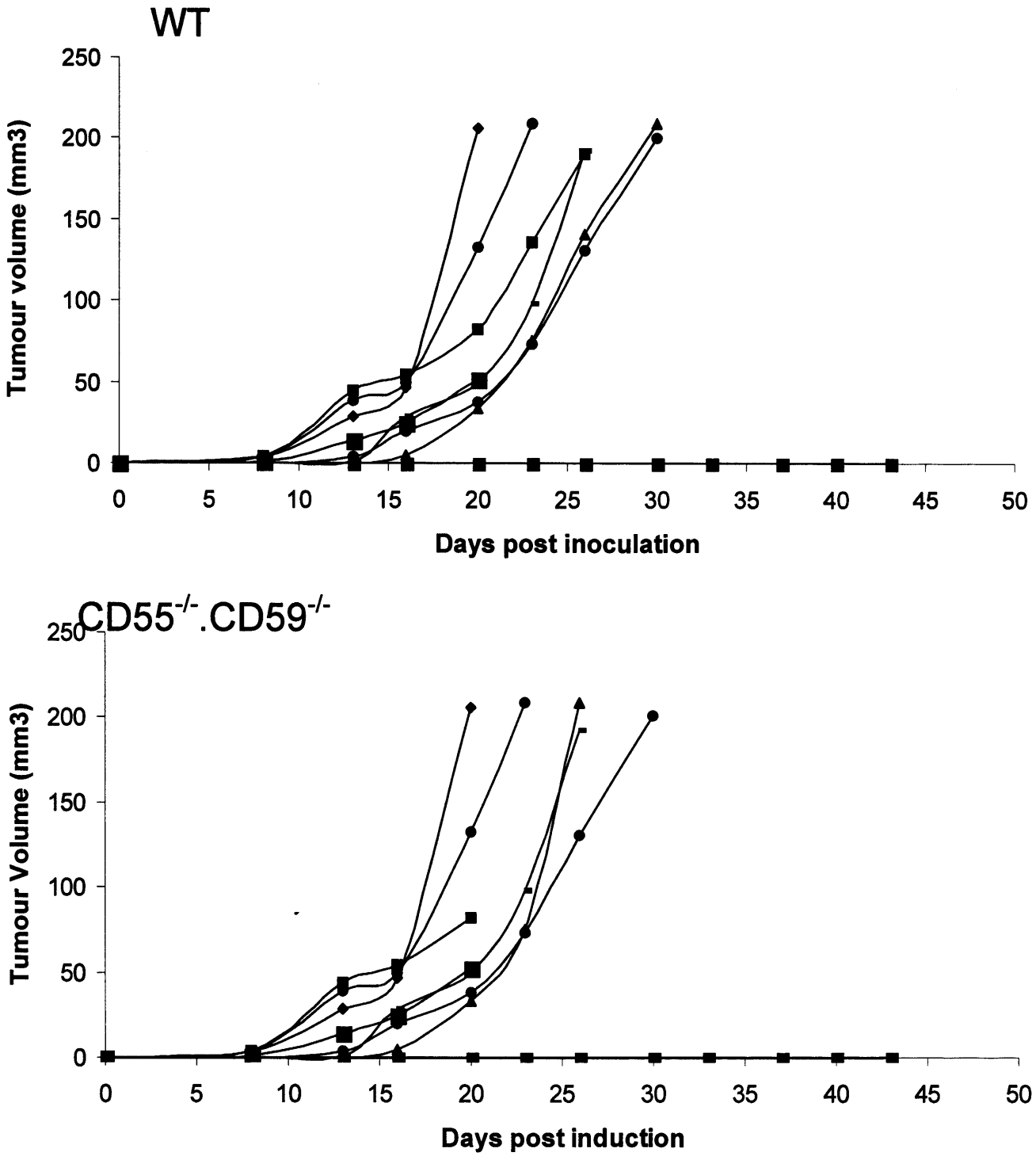


Figure 4.16: Growth of K6 fibrosarcoma in WT and $CD55^{-/-}.CD59^{-/-}$ male mice
Eight WT and $CD55^{-/-}.CD59^{-/-}$ mice were inoculated with 10^5 K6 cells. Mice were injected on day 1 with 100μL tumour cells on the flank and monitored regularly with tumour sizes measured. Each line is representative of one individual mouse. Mice were sacrificed when tumour reached $200mm^3$. Data shown (n=8) are representative of 3 separate experiments.

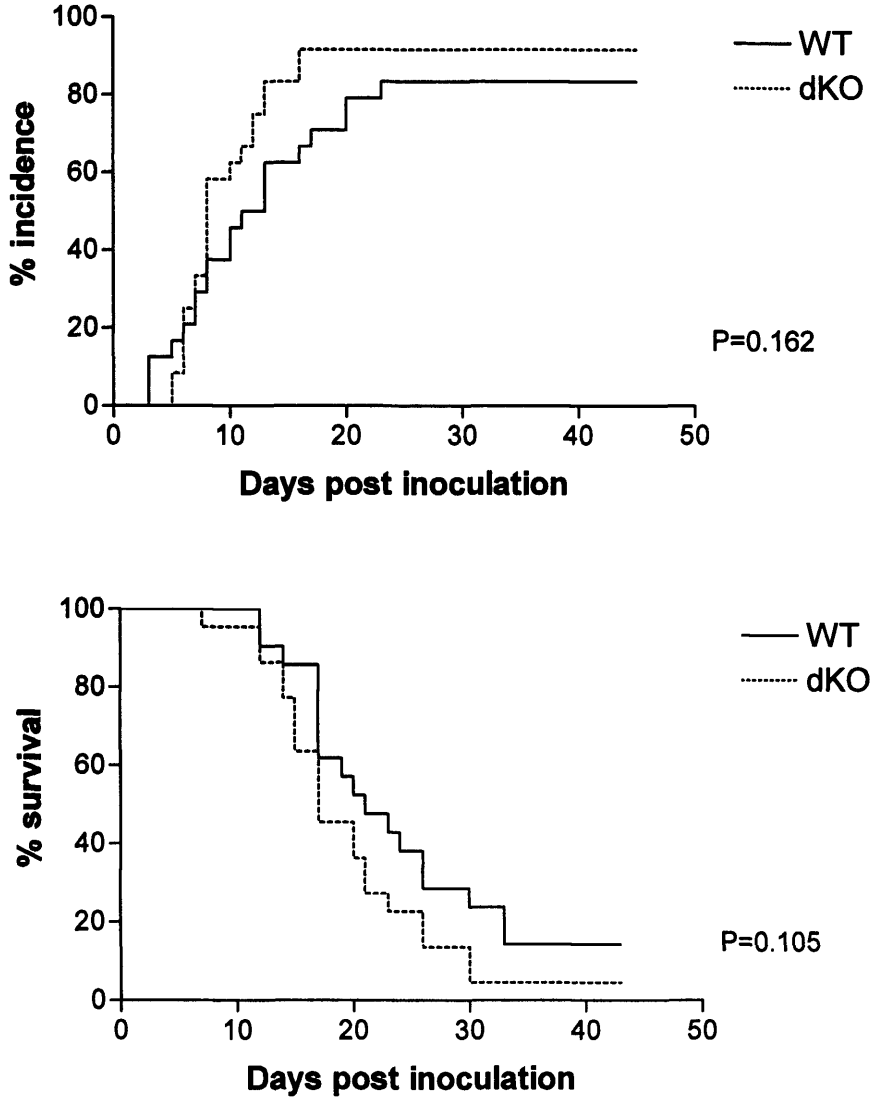


Figure 4.17: K6 survival and incidence in WT and CD55$^{-/-}$.CD59$^{-/-}$ male mice
Pooled data from WT male (n=24) and CD55$^{-/-}$.CD59$^{-/-}$ male (n=24) .Mice were inoculated with 10^5 K6 cells, sub-cutaneously into the left flank. K6 incidence occurs when a visible tumour is observed, while survival events are marked when tumour reached pre-determined volume (200mm^3). Differences between the two groups was analysed using the log-rank test. The effects were found not to be statistically significant ($p>0.05$).

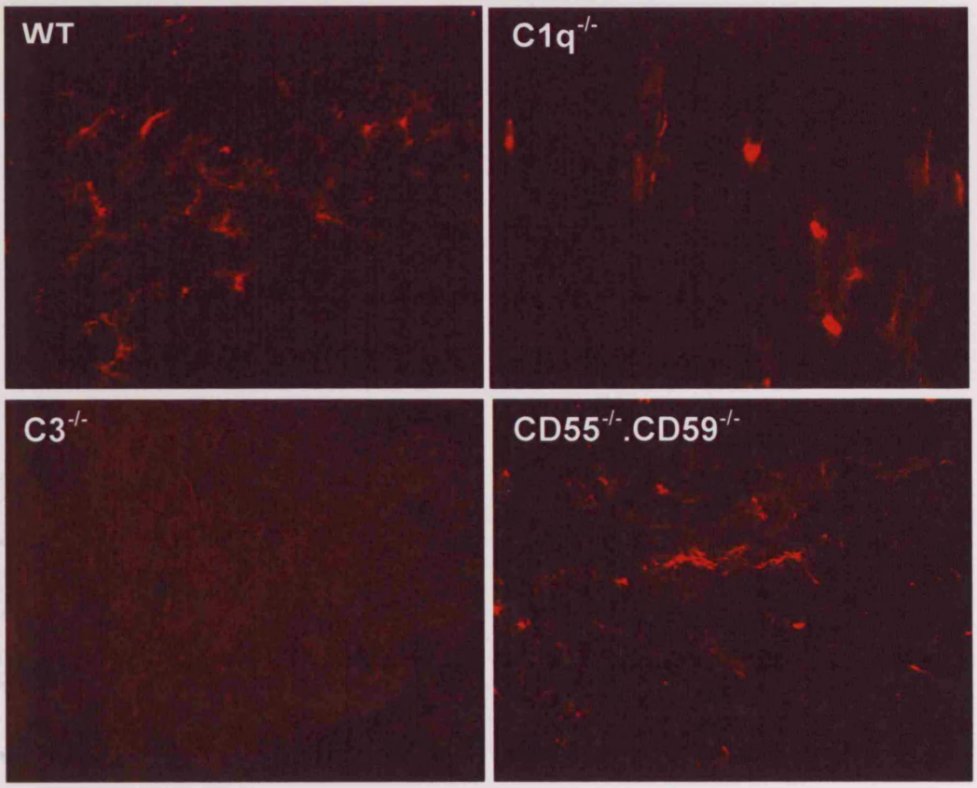


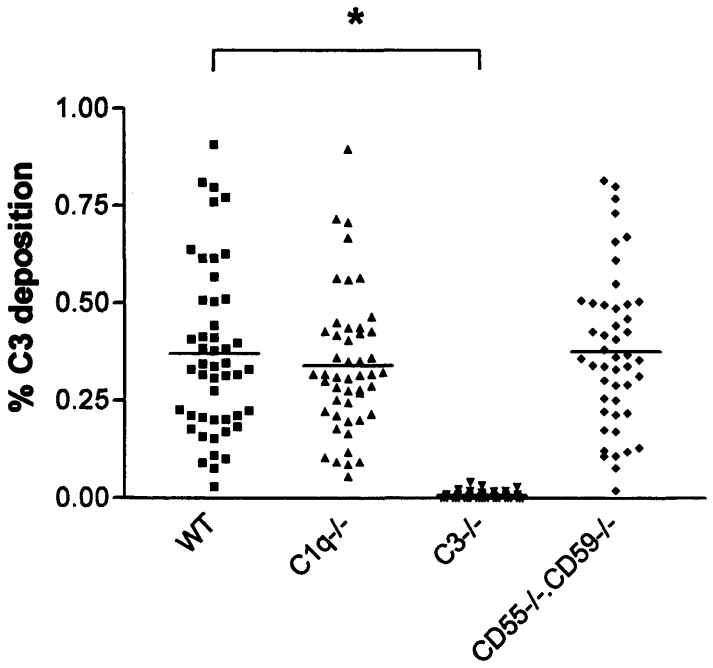
Figure 4.18: C3b/iC3b deposition on K6 tumour sections
C3 cleavage and deposition on K6 tumours grown in WT and KO hosts was assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of diseased tissue (not shown) using a rat monoclonal anti-mouse C3/C3b/iC3b antibody. Alexa-fluor 594 conjugated donkey anti-rat secondary antibody was used to detect fluorescence. Images shown are representative sections from each tumour (n=6). Tissue from each genotype of tumour was also stained using an isotype control antibody, negative staining was found.

C1q deficiency, host C3 must be derived from alternative or MBL pathways. However, it is possible that K6 synthesises and secretes C3, leading to tumour derived C3b deposition on its own surfaces. However the lack of C3b deposited on K6 in $C3^{-/-}$ mice suggests this is not the case and that deposition on other tumour tissues is derived from the host animal. In order to quantify levels of C3 fragment deposition on K6 cells, sections were analysed using Image Pro Analyser software. Use of this software allows a fluorescence threshold to be set, above which signal is marked as positive, The % area stained positive can then be calculated per random camera frame. Data shown in Figure 4.19 show comparable and consistent levels of C3b/iC3b deposition detected on K6 tissue in WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ hosts. All tissues examined showed signs of C activation except those grown in $C3^{-/-}$ animals where no positive signal for C3b/iC3b was observed, this was found to be statistically significant compared to staining in WT group ($p<0.001$).

Data showing C3b/iC3b deposition on K6 tissues in C5-neutralised and isotype control treated mice are shown in Figure 4.20. Quantitation of C3b/iC3b deposition again revealed no differences between the groups indicating similar levels of C activation and subsequent deposition of C3 fragments in these groups (Figure 4.21).

4.2.7. C9 deposition on K6 tumour tissue

Deposition of C9 also represents an informative marker of C activation, and the presence of C9 on membranes indicates activation of the terminal pathway thus providing evidence of C5a release and MAC deposition. Both these effects are significant and may have important effects on tumour cells, and various other cells in the tumour microenvironment e.g. immune effector cells. Effects associated with C5a release and MAC deposition are described in sections 1.2.1 and 1.12 respectively. Figure 4.22 shows a representative slide from a K6 tumour section grown in each of WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}$.$CD59^{-/-}$ genotypes. Each picture is representative of 8 sample image slices taken from 6 separate tumours inoculated into each mouse strain. Evidence



	Mean ± SEM
WT	0.372 ± 0.031
$C1q^{-/-}$	0.341 ± 0.026
$C3^{-/-}$	0.006 ± 0.001
$CD55^{-/-}.CD59^{-/-}$	0.378 ± 0.029

Figure 4.19: Immunohistochemical analysis of C3b/iC3b deposition
Percentage area stained positive for C3b/iC3b deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test. * indicates $p<0.05$.

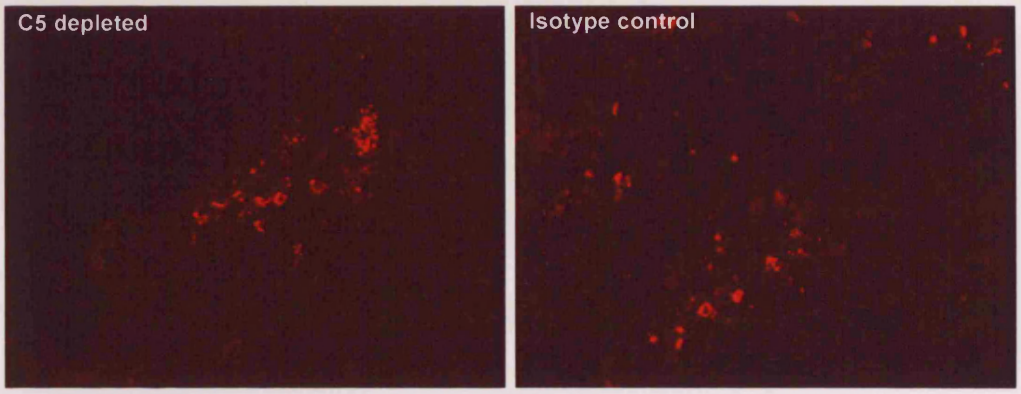
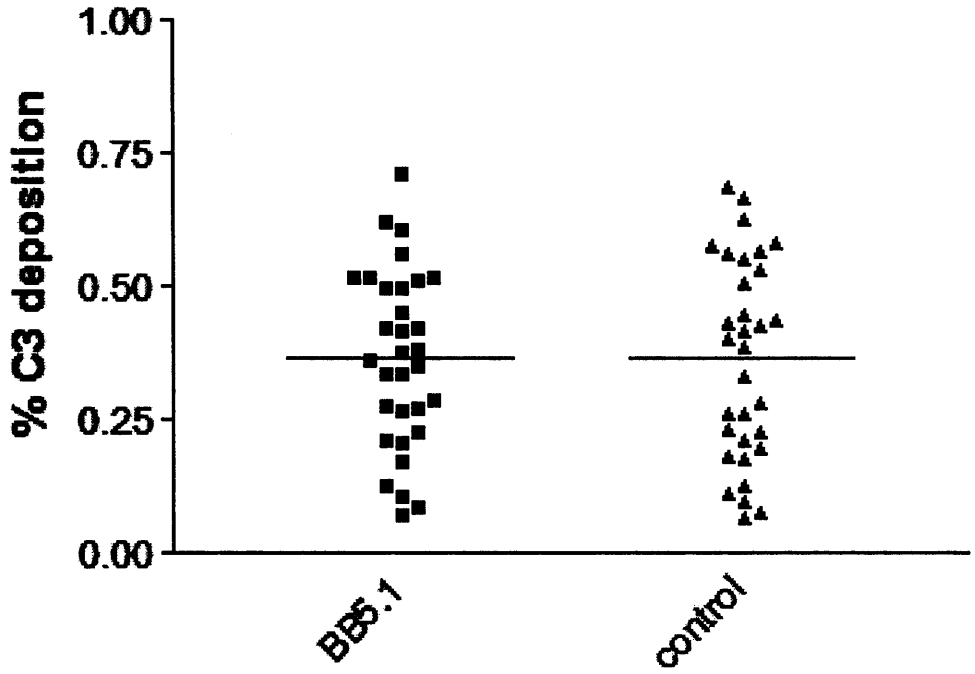


Figure 4.20: C3b/iC3b deposition on K6 tumour sections following C5 depletion
C3 cleavage and deposition on K6 tumours grown in WT and C5-depleted hosts was assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of diseased tissue (not shown) using a rat monoclonal anti-mouse C3/C3b/iC3b antibody. Alexa-fluor 594 conjugated donkey anti-rat secondary antibody was used to detect. Images shown are representative sections from each tumour (n=6). Tissue from each genotype of tumour was also stained using an isotype control antibody, negative staining was found.



	Mean ± SEM
C5-depleted	0.368 ± 0.029
control	0.365 ± 0.033

Figure 4.21: Immunohistochemical analysis of C3b deposition following C5 depletion
Percentage area stained positive for C3b/iC3b deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test.

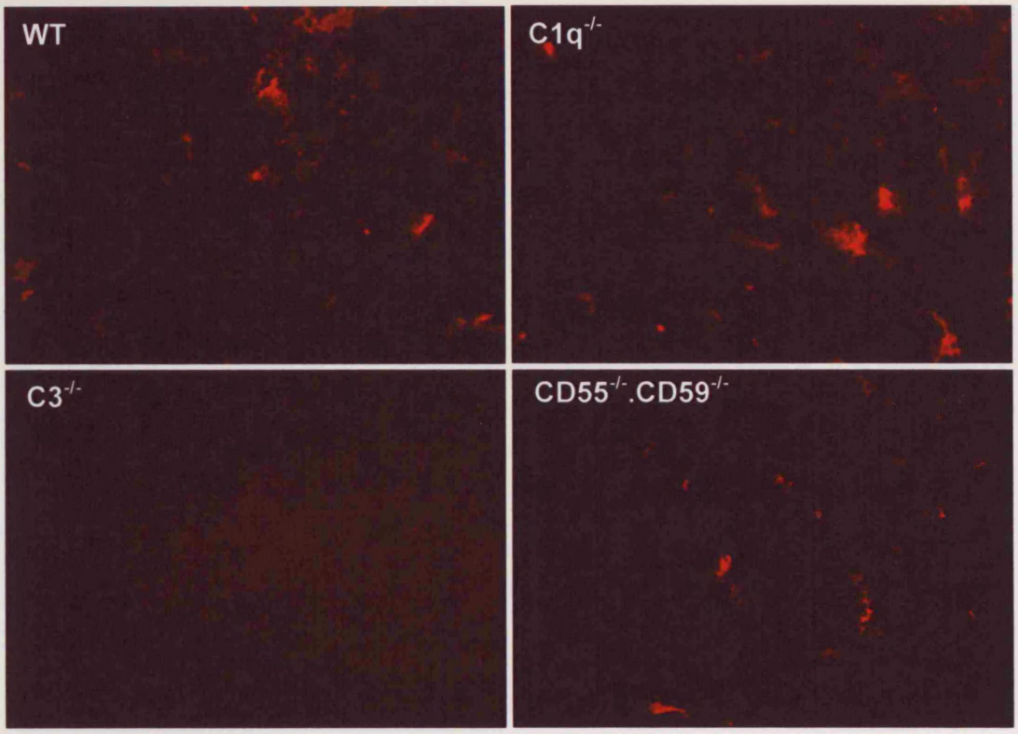
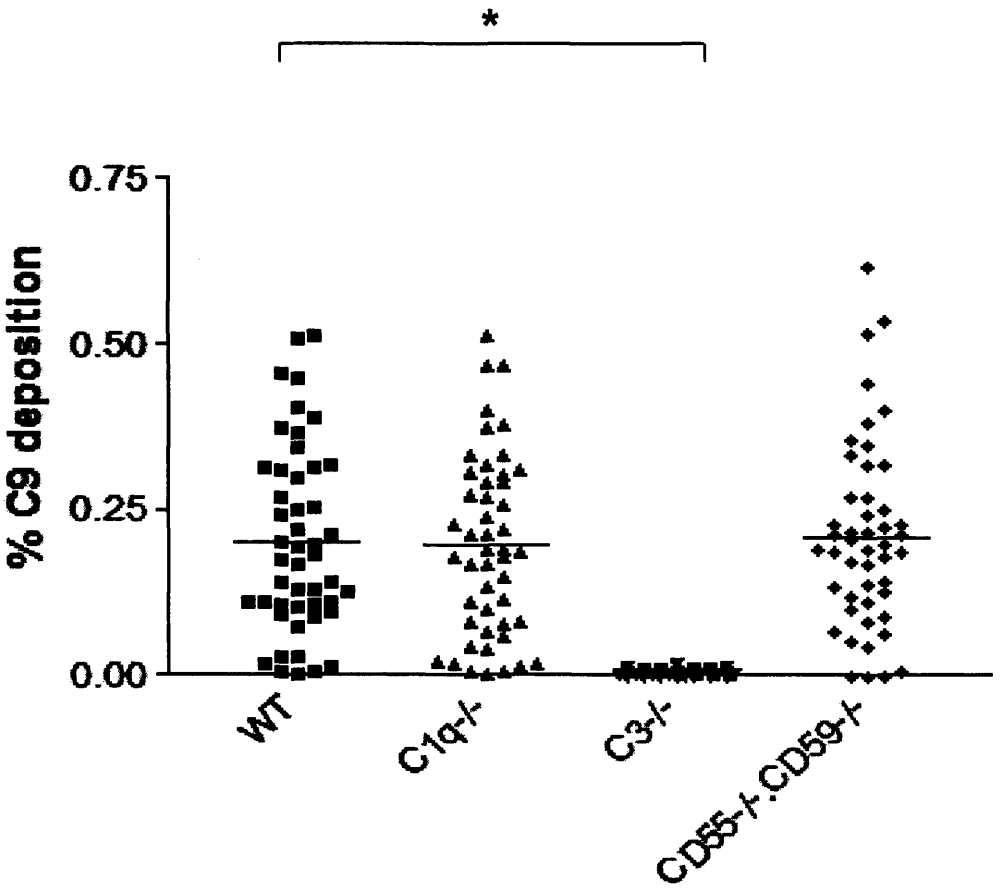


Figure 4.22: C9 deposition on K6 tumour sections

C9 deposition on K6 tumours grown in WT and KO hosts was assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of diseased tissue (not shown) using a rabbit anti-rat C9 antibody. Alexa-fluor 594 conjugated donkey anti-rat secondary antibody was used to detect fluorescence. Images shown are representative sections from each tumour (n=6).

for C9 deposition was observed on each tumour section analysed, with the exception of those grown in $C3^{-/-}$ hosts. This result is expected given the accepted mechanisms for initiation of the terminal pathway (see Figure 1.1) and indicates that C9 deposited on other K6 tumours is generated via C3-dependent mechanisms. These data were analysed as described for C3b/iC3b deposition in order to obtain quantifiable data and compare levels of C9 deposition between groups. Figure 4.23 shows no statistically significant difference in fluorescence per tumour frame between WT and $C1q^{-/-}$ ($p=0.817$) or WT and $CD55^{-/-}.CD59^{-/-}$ groups ($p=0.789$) with mean % deposition values shown to be equivalent between groups. By contrast, a statistically significant difference was observed between K6 tumours grown in WT and $C3^{-/-}$ hosts ($p<0.001$). This finding provides conclusive evidence for the C3-dependent generation of C9 deposition and suggests that the various effects associated with MAC deposition may be relevant in this model.

Data shown in Figure 4.24 are representative of C9 staining on K6 tumours grown in C5-neutralised mice, and those treated with an isotype control Ab. Representative images shown reveal C9 deposition in WT (isotype control) group, but no deposition in C5-neutralised hosts. This finding is confirmed following quantitation analysis (Figure 4.25) which shows a statistically significant difference in C9 deposition between WT and C5-neutralised groups ($p<0.001$). Cleavage of C5 represents the initiation step for activation of the terminal pathway, and the lack of C9 deposition in C5-neutralised hosts provides strong evidence against activation of the terminal pathway in these mice. Consequently, negligible MAC deposition is observed, demonstrating that C5 neutralisation was effective and that no C5a is released following activation of C by tumour cells.



	Mean ± SEM
WT	0.202 ± 0.020
$C1q^{-/-}$	0.196 ± 0.019
$C3^{-/-}$	0.004 ± 0.001
$CD55^{-/-}.CD59^{-/-}$	0.209 ± 0.022

Figure 4.23: Immunohistochemical analysis of C9 deposition

Percentage area stained positive for C9 deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test. * indicates $p<0.05$.

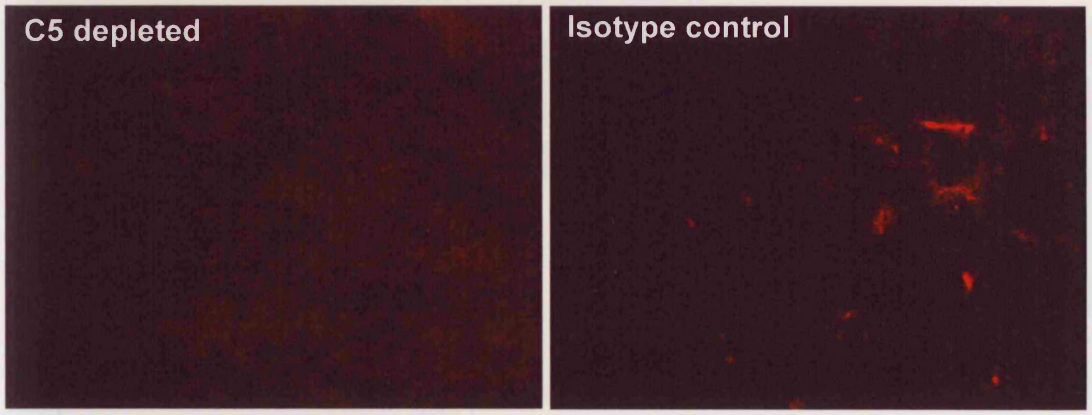
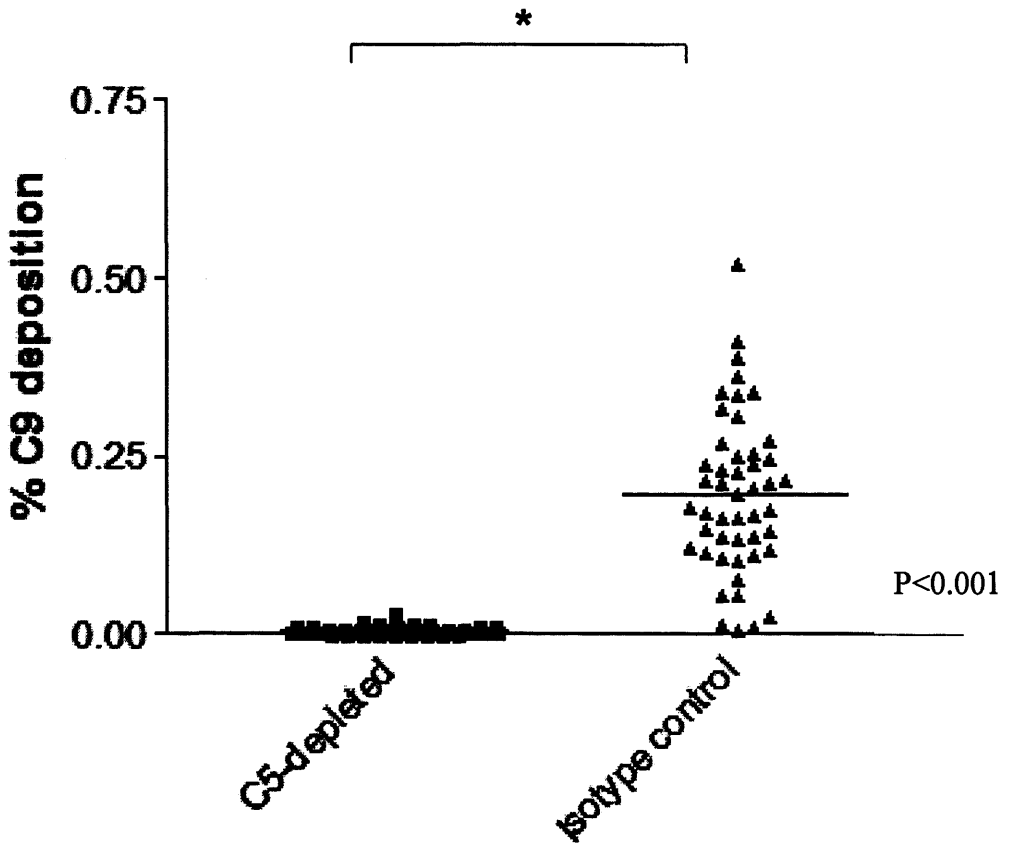


Figure 4.24: C9 deposition on K6 tumour sections following C5 depletion
C9 deposition on K6 tumours grown in WT (isotype control treated) and C5-depleted hosts was assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of diseased tissue (not shown) using a rabbit polyclonal anti-rat C9 antibody. Alexa-fluor 594 conjugated goat anti-rabbit secondary antibody was used to detect fluorescence. Images shown are representative sections from each tumour (n=6).



	Mean ± SEM
C5-depleted	0.005 ± 0.001
control	0.197 ± 0.016

Figure 4.25: Immunohistochemical analysis of C9 deposition following C5 depletion
Percentage area stained positive for C3b/iC3b deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test ($p<0.001$). * indicates $p<0.05$.

4.2.8. $CD4^+$ cells infiltrate K6 tumour tissue

It has previously been reported that $CD4^+$ T-cell responses are enhanced in mice lacking CD55 (Liu et al. 2005) and CD59 (Longhi et al. 2005). For this reason, differences in $CD4^+$ infiltrating cells may be evident in mice of different genotypes. Infiltration of $CD4^+$ cells, the majority of which are T-cells (Awen Gallimore, personal communication), into K6 tumours was assessed by immunohistochemistry. $CD4^+$ cells were observed in all tumour tissues analysed and were visible as a defined ring of fluorescence (Figure 4.26). In order to assess differences between groups, numbers of infiltrating cells were counted (8 frames per tumour, n=6). The $CD4^+$ infiltrate appears slightly lower in $C1q^{-/-}$ and $C3^{-/-}$ hosts than in WT hosts. However, statistical analysis revealed that differences were not significant as evidenced by p-values of 0.120 and 0.086 respectively (Figure 4.27).

Similar data gathered from C5-neutralised and isotype control treated host mice revealed no difference between these groups with similar levels of infiltration as shown in Figures 4.28 and 4.29.

4.3. Discussion

The aims of the work described in this Chapter were to identify differences in tumour growth as a result of deficiencies in specific C proteins. Deficiencies in C1q, C3 and double deficiency in CD55 and CD59 did not cause significant observable differences in K6 growth or survival of inoculated mice. Despite the lack of classical pathway activation in $C1q^{-/-}$ mice, and abrogation of all three activation pathways in $C3^{-/-}$ mice, no pro-tumour effects were observed through impaired immune responses. If C was contributing significantly to immune-mediated control of K6 growth, either directly or through related effects, differences in tumour development would be expected. Therefore, these data suggest that C plays little if any role in controlling growth of the established fibrosarcoma line K6 *in vivo*. Additionally, the hypothesis that C

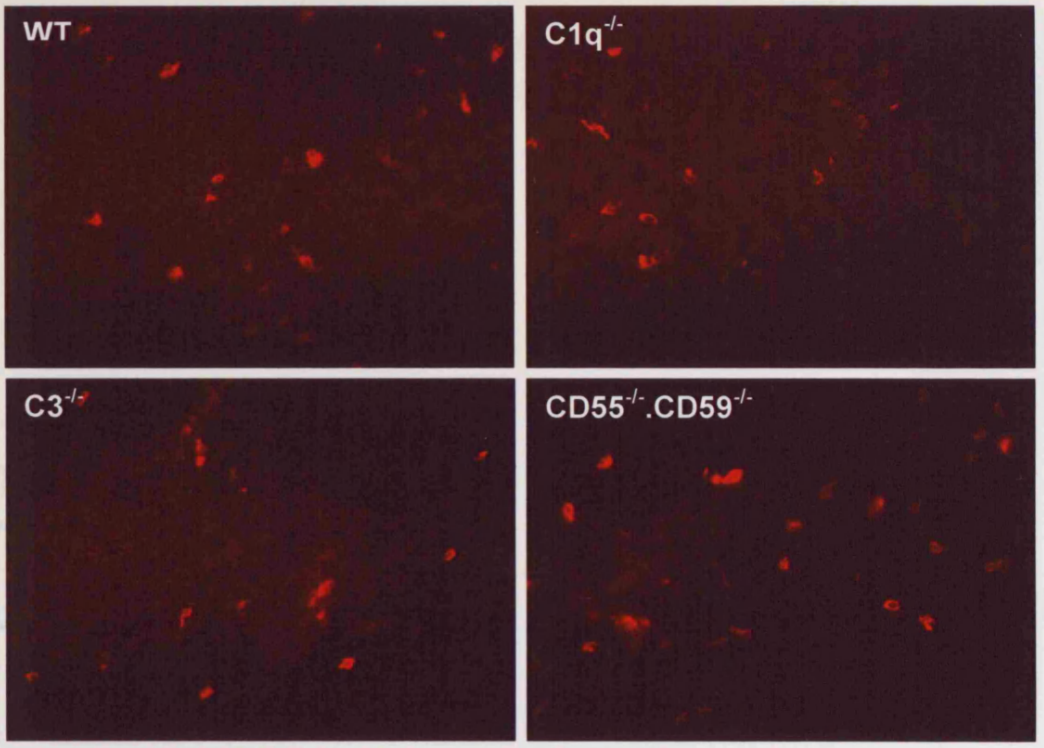


Figure 4.26: CD4+ Cell infiltrate in K6 tumour sections
CD4+ cells in K6 tumours were assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of spleen (not shown) using a rat monoclonal anti-mouse CD4 antibody. Alexa-fluor 594 conjugated donkey anti-rat secondary antibody was used to detect. Images shown are representative sections from each tumour (n=6). Tissue from each genotype of tumour was also stained using an isotype control antibody, negative staining was found. CD4+ cells were counted by eye.

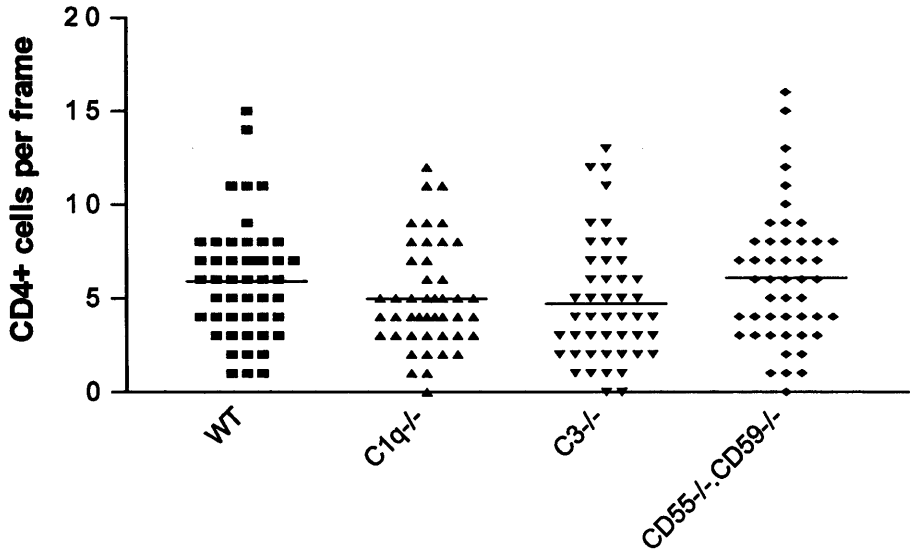


Figure 4.27: Immunohistochemical analysis of CD4+ infiltrate
Numbers of CD4+ cells per section were counted, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test.

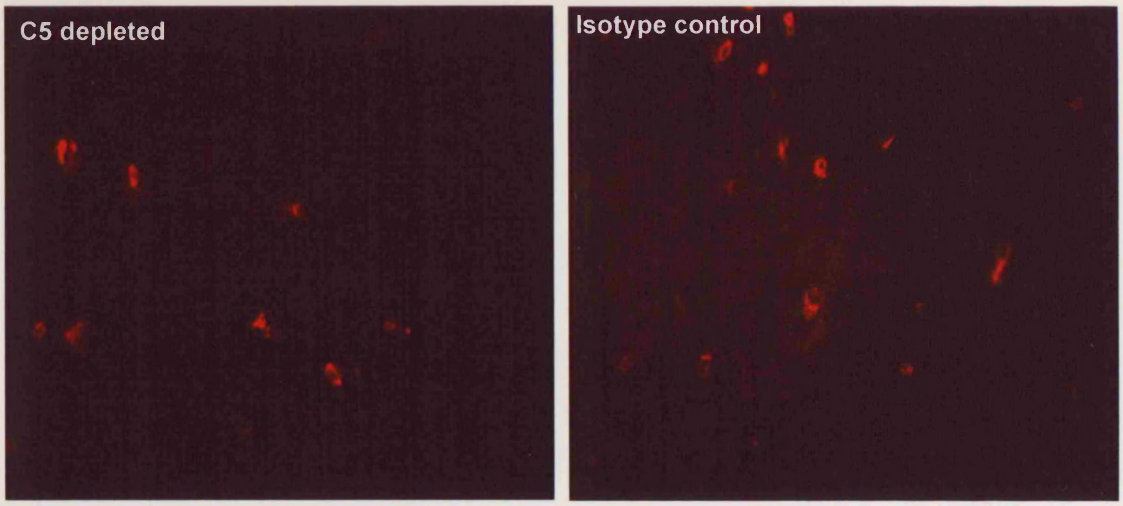


Figure 4.28: CD4+ Cell infiltrate in K6 tumour sections
CD4+ cells in K6 tumours from WT and C5-depleted mice were assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of spleen (not shown) using a rat monoclonal anti-mouse CD4 antibody. Alexa-fluor 594 conjugated donkey anti-rat secondary antibody was used to detect. Images shown are representative sections from each tumour (n=4). Tissue from each genotype of tumour was also stained using an isotype control antibody, negative staining was found.

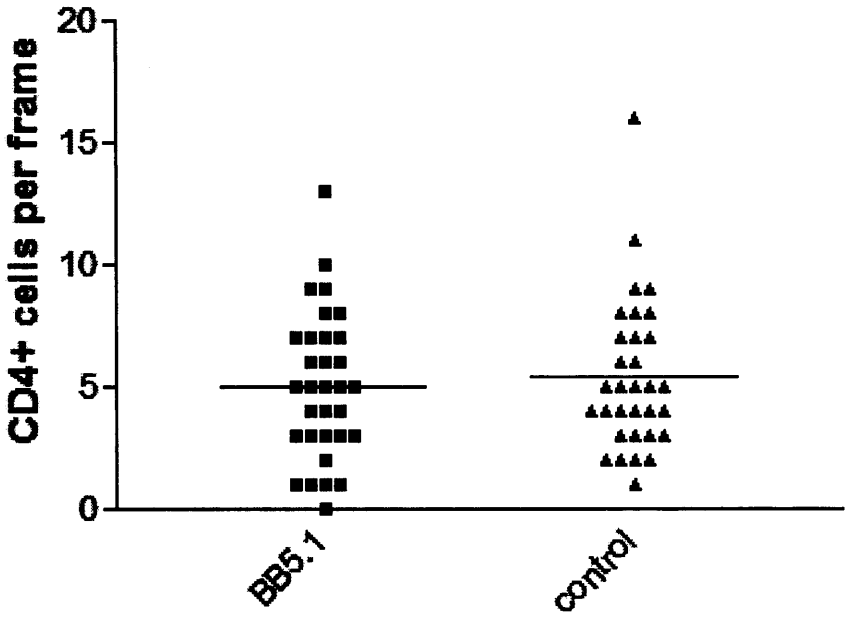


Figure 4.29: Immunohistochemical analysis of CD4+ infiltrate

Numbers of CD4+ cells per section were counted, 8 frames were assessed per tumour with 4 tumours used per WT and C5-depleted group. Data were analysed using a 2 tailed, unpaired t-test.

may aid tumour proliferation was also not supported by these data as no differences were observed in the absence of C1q or C3. If either protein was able to significantly drive K6 proliferation, faster growth rates, and lower survival would be expected in these groups.

However, K6 is a highly aggressive fibrosarcoma, arising shortly after inoculation and proliferating rapidly. As a result subtle differences between groups may be masked due to the rapid proliferation rates, and potential disparities in tumour growth between mouse strains may be hidden. As this tumour cell line takes just days, typically between 10-15 days, to progress from a barely visible tumour, to the mouse being sacrificed, large group sizes would be needed to dissect out differences between tumour growth patterns in differing genotypes. In order to investigate whether lower tumour challenges slowed tumour growth, the number of K6 cells inoculated into WT mice was varied. Differences in tumour challenge administered did affect number of mice developing a tumour, with lower K6 titres corresponding with lower tumour incidence. However, no differences in proliferation rates were observed as a result of administering 10^4, 10^5 or 10^6 K6 cells into WT mice (data not shown). Alternatively, a less aggressive cell line could be used to allow more time for potential differences to be visualised. This approach is detailed in Chapter 6.

Despite the aggressive nature of K6, initial data did suggest a protective effect mediated by administration of a blocking mAb against mouse C5. As described above, there were complications associated with this experiment. The presence of endotoxin in the antibody preparation and lack of control immunoglobulin in a preliminary experiment could both contribute to the observed effects. Endotoxin is known to stimulate large scale immune reactions and cytokine release (Cooke et al. 2002) which could well contribute to the immune response generated in these mice, unrelated to the neutralisationof C5. Endotoxins are known to strongly stimulate macrophages and neutrophils (Kirkland et al. 1993) and this heightened immune sensitivity is likely to alter the immune response to tumour inoculation in these animals.

However, further controlled experiments corroborated the previous finding and neutralisation of C5 *in vivo* was shown to hinder tumour growth (p=0.045). Cleavage of C5 releases a potent anaphylatoxin C5a with powerful roles in the inflammatory response. Inflammation has long been considered a powerful driving mechanism facilitating tumour growth (Coussens and Werb, 2002), and the lack of this inflammatory signal may be slowing tumour growth in C5-neutralised animals. However, recent data by Markiewski *et al* (2008) has shown that C5a can contribute to suppression of the immune response through a specific immunosuppressive mechanism. In this study, enhanced tumour growth was linked to recruitment of Myeloid Derived Suppressor Cells (MDSCs) known to express high levels of C5aR (Guo and Ward 2005). These suppressive cells can be stimulated to produce high levels of reactive oxygen species (ROS) (Corzo et al. 2009). The interaction of C5a and C5aR was demonstrated to be an important event with ROS also shown to inhibit $CD8^+$ T-cell responses (Kusmartsev et al. 2004). The same study found that $CD8^+$ T-cell responses, thought to contribute significantly to anti-tumour immunity, were enhanced after blocking of C5aR (Swann and Smyth 2007). Thus, the authors hypothesise a key role for C5a in suppressing immune responses through modulation of $CD8^+$ T-cell activity.

The role of C5a as a pro-tumour effector molecule is not supported by other data from C3-/- animals. In this case, no C5 cleavage is expected as neither the classical nor alternative pathway C5 convertase can be assembled meaning that downstream effects are not possible. A similar protective effect to C5 neutralisation would initially be expected through similar dampening of the inflammatory response and abrogation of associated pro-tumour effects. One recent report describes generation of C5a in a C3 independent manner (Huber-Lang et al. 2006) yet the *in vivo* relevance of this pathway is unclear. Evidence against this hypothesis is also provided in (Figures 4.22 and 4.23) with the absence of deposited C9 in $C3^{-/-}$ mice. This observation indicated that no MAC is deposited in these mice and that MAC deposition in WT hosts requires C3b deposition. The lack of C5 convertase assembly and terminal pathway activation in $C3^{-/-}$ mice also suggests that C5a release in these mice will be reduced or absent in the tumour microenvironment. The lack of a

protective effect in $C3^{-/-}$ mice may be a result of numerous mechanisms related to C activation and deposition. As discussed in Chapter 1, C3 has huge potential to interact with a wide range of immune cell types, any of which could be contributing to immune control of tumours in this model through significantly impaired opsonisation mechanisms. In this model, the near complete abrogation of C is these animals will have numerous effects in terms of inflammation and both innate and adaptive immunity. The hypothesis that C plays a dual role in tumour formation and proliferation remains valid with subtle differences unlikely to be observed using such an aggressive cell line. The effects of a 'double-edged sword' role played by C could be cancelled out in terms of its anti- and pro-tumour effects culminating in equivalent growth rates and survival. In depth evaluation of evaluating cell types into K6 tumours grown in different host genotypes may elucidate contributory mechanisms and allow further investigation into tumour growth in these mice.

The use of a neutralising mAb has a major advantage over the use of knock-out animals as mice used are on identical genetic backgrounds and littermate controls may be used. In this way, the only difference between groups is the parameter in question. Experiments described in this Chapter investigated the effect of C5 protein on tumour take. Although every care was taken to back-cross onto the C57/BL6 background (at least 10 generations), $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice utilised in this study will have subtle genetic differences which may manifest as minor histocompatibility issues and lead to altered tumour growth. No differences were observed in K6 growth between WT and $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ hosts.

A non-significant trend was observed suggesting predisposition of $CD55^{-/-}$ $CD59^{-/-}$ mice to slightly faster tumour growth. However, differences were difficult to identify and found not to be statistically significant. Further studies would be required to validate this observation while reasons underlying the trend remain unclear and complex. Inflammation is known to drive tumour progression and these animals, with impaired ability to control C attack, would seem likely to have enhanced chronic inflammation. However, as discussed in Chapter 1, both these proteins play important roles outside C regulation and

are implicated in T-cell responses. Deficiencies in either CD55 and CD59 have been shown to enhance T-cell responses, reviewed by (Longhi et al. 2006) and are thought to exhibit 'exaggerated' T-cell activities. However, data described indicate that K6 tumours grew as well in mice deficient in CD55 and CD59 as WT mice thus any enhanced T-cell responses were ineffective against K6 growth. An alternative effect could be mediated through lack of CD59. Expression of CD59 has been shown previously to enhance the NK-cell response (Omidvar et al. 2006). It is possible that defective NK responses in these mice allow tumour cells to escape immune killing more readily and contribute to the decreased survival observed in these mice.

Another important consideration is that injection of WT tumour cells into a knock-out mouse could stimulate an immune response to the corresponding protein encoded by the knocked-out gene. For example CD55 or CD59 expressed on K6 cells may raise an immune response in $CD55^{-/-}.CD59^{-/-}$ mice not tolerised to these proteins. However, no evidence to support additional immune responses to these cells is provided by these studies with K6 cells growing comparably in WT and $CD55^{-/-}.CD59^{-/-}$ hosts. Additionally, there is evidence to support C1q (Al-Adnani and McGee 1976) and C3 (Garred et al. 1990) secretion by fibroblasts, also with potential to raise antibody responses and trigger enhanced cytotoxicity. These proteins may be recognised as foreign and therefore elicit an immune response through Ab production or CD8+ T-cell responses. Data in Chapter 3 showed that neither CD55 nor CD59 is expressed on K6 cells *in vitro* while C3 was not detected by ELISA or RT-PCR techniques. As shown in Figure 4.23, no C3 was detected in/on K6 cells grown in $C3^{-/-}$ hosts. This observation suggests that K6 fibrosarcomas do not store C3 intra-cellularly, and do not exhibit tumour derived C3b/iC3b deposited on cell surfaces. These data also suggest that C3b/iC3b observed on other tumour sections is derived from activation of the host C system. Whilst non-transformed fibroblasts, and several tumour cell types have been shown to secrete C3 *in vivo* (Katz, Revel and Strunk 1989, Jean et al. 1997), induction of tumours by 3-MCA produces highly heterogeneous tumour cell populations with diverse phenotypes (Pimm, Embleton and Baldwin 1980). K6 is an established homogeneous line and although no evidence of C3

secretion is observed, that is not to say this is a common feature of all fibrosarcoma cells.

Similar levels of C3b/iC3b were found deposited in all host animals except, as expected, those deficient in C3. Given the similar haemolytic capabilities of WT and $CD55^{-/-}.CD59^{-/-}$ mice described in this Chapter, similar levels of activation and deposition of activation fragments would be expected in these groups. However, the observation that K6 cells exhibited comparable levels of C3b/iC3b deposition in $C1q^{-/-}$ mice is interesting and suggest that the classical pathway is not responsible for the majority of activation and point to an important role for MBL or alternative pathway activation by K6 cells. Since C5 cleavage occurs downstream of C3 in all activation pathways (see Figure 1.1), no difference was expected in activation and deposition between C5-neutralised and control groups. This was confirmed by immunohistochemical analysis.

Scant data are available which describe levels of C deposition on normal i.e. non-transformed fibroblast cells *in vitro*. However, a study on normal human skin showed that fibroblasts could be identified in excised tissue biopsies, and that C3 deposition was only observed following *in vitro* incubation with high concentrations of sera (Schuler et al. 1982). Addition of C4-deficient sera resulted in samples which stained negative for C3 activation products. Fibroblasts excised from normal and C3-deficient patients both showed no deposition of C3 products after being transferred to cover slips and examined *in vitro* (Fishelson et al. 1999). A further study in mice has also showed that C activation and C3 deposition was observed on virus-infected cells but not on non-virus infected cells (Miyazawa et al. 1987). Cells examined in this study were not fibroblasts, but are of the same mesodermal origin, in this case smooth muscle cells.

Immunohistochemical analysis of C9 deposition on K6 tumours grown in WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice revealed no differences, as previously demonstrated for C3b/iC3b deposition. These findings are consistent given the requirement for C3b deposition for formation of both classical and

alternative C5 convertases. Thus, equivalent levels of C3b deposition would be expected to generate equivalent levels of MAC deposition. Also consistent with previous findings was the absence of deposited C9 on tumours grown in $C3^{-/-}$ mice. These data suggest that no C3b deposition, C3a / C5a release or MAC deposition is occurring in these hosts. Therefore, the previous demonstration that K6 tumours grew comparably in WT and $C3^{-/-}$ hosts is particularly important and suggests that the effects associated with these important consequences of C activation are neither contributing to tumour immunosurveillance nor to promotion of tumour growth in this model.

No differences were observed following analysis of $CD4^{+}$ infiltrating cells into K6 tumours. Whilst this marker provides a preliminary assessment of adaptive immune responses to tumour inoculation, further work would be needed to address T-cell phenotype and activity of these cells. However, the observation that T-cell numbers were not noticeably higher in $CD55^{-/-}.CD59^{-/-}$ animals is important given the recent roles associated with these proteins expressed on T-cells. The short timespan for these experiments may prevent a significant difference from developing, while stronger responses may be elicited without excessive recruitment of cells.

Data presented in this Chapter provide a primary investigation of how C may affect tumour growth *in vivo*. As previously discussed, use of an established and aggressive tumour line allows only a brief timescale for differences to become evident. However, some significant effects were observed indicating a protective effect of C5 neutralisation in hosts allowing enhanced control of tumour growth. This finding contradicts dogma whereby a compromised C response would be expected to result in reduced control over tumour growth. A better understanding of the role of C over a longer timescale and in the developing tumour may provide an improved model for assessing the relative roles of C deficiencies. The use of a chemical carcinogen allows such investigation and is detailed in the following Chapter.

5. The effect of complement deficiency on 3-methylcholanthrene (3-MCA) induced tumours

5.1. Introduction

As discussed in Chapter 1, C regulators are strongly implicated in protection of tumours from C-mediated lysis. Additionally, emerging evidence supports a role for C activation and deposition in tumour development. Investigation of the respective roles of certain C proteins (i.e. components/regulators) can be achieved by administration of the chemical carcinogen 3-MCA in mice. Fibroblasts are recruited to the site of injection and are subjected to the mutagenic properties of the carcinogen. Multiple mutations arise and *de novo* tumour development over a period of months can be assessed. Numerous studies have successfully used this model to dissect effects of various immune functions leading to enhanced tumour proliferation or rejection. Such studies include the respective roles of Interferon-γ (Qin et al. 2002), cytotoxic T-cells (DeLustro and Haskill 1978) and NK-cells (Crowe, Smyth and Godfrey 2002) in 3-MCA tumourigenesis. The role of C has not been investigated in this model.

The experiments presented in this Chapter were designed to assess whether deficiency of various C components or regulators in host mice, affected *in vivo* tumour development. Induction of tumours via chemical carcinogenesis varies considerably between different animal housing units with environmental factors, mouse strain and dose of carcinogen being important. Generally, tumours arise and develop over a period of 2-6 months with a plethora of factors affecting their development. Mice deficient in C1q, C3 and lacking both CD55 and CD59 were used to examine the respective roles played by these proteins in tumour formation and proliferation. The hypothesis that a compromised C system would lead to reduced control of tumour growth illustrated by higher incidence and faster proliferation was tested. It was further hypothesised that tumours induced in mice deficient in two potent CReg would exhibit reduced protection against C-attack thereby resulting in

lower incidence and slower proliferation. Mice of differing genotypes ($C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$) were administered MCA alongside WT control animals. Tumour incidence and progression were monitored over several months with mice assessed weekly.

In addition to tumour incidence and survival of mice, further understanding of C-related effects was sought via post-excision analysis of tumour tissue. Fibrosarcomas were analysed for regulator expression and C deposition. Examination of tumour tissues was performed using flow cytometry on live cells and via immunohistochemical techniques on frozen tissue sections. Tumours induced in KO mice were compared to those induced in age-matched wild type mice. These mice provided a standard against which tumours lacking C-related proteins were compared. Additionally, since WT mice were injected alongside every KO group, these mice acted as a control against variations in tumour growth due to changes in environmental conditions. As reviewed by (Blankenstein and Qin 2003), considerable variation is observed between studies performed in different housing conditions and between strains of mice. As such, all mice were on identical C57 Bl6 backgrounds, backcrossed for at least 10 generations and controlled against WT animals for each batch of MCA injections.

5.2. Results

5.2.1. Susceptibility of male and female WT mice to 3-MCA-induced tumours

Tumours induced in this chemical carcinogenesis model arise at varying times depending on 3-MCA dose, strain of mouse and environmental housing factors. Typically, WT C57Bl/6 mice develop tumours between 90-150 days post injection of 400µg 3-MCA (Gareth Betts, personal communication, November 2005). Once these tumours reached 1cm diameter, mice were sacrificed and tumours excised.

In order to evaluate differences attributable to deficiency in C proteins, experiments must be controlled with WT mice. An important variable to consider is gender of mice used in these experiments and it was first deemed necessary to investigate whether differences exist in 3-MCA tumour induction between male and female mice. As described in Chapter 2, mice were administered 400μg 3-MCA sub-cutaneously in the hind leg. Initially, male and female WT mice were injected simultaneously with tumour growth monitored over several months. Figure 5.1 shows survival data gathered from 16 male and female WT mice. No statistically significant differences in tumour induction were observed between male and female WT mice ($p=0.23$). However, a trend for higher tumour incidence was observed in male mice. In males, 0 from 16 mice remained tumour free compared to 5 from 16 female mice at the end of the experiment. This suggests that male mice are more susceptible to tumour development, a trend that would likely become significant given larger group sizes. Consequently, it was deemed inappropriate to compare male and female mice in the same group in follow-on experiments. Due to the enhanced C activity previously observed in male mice (Figure 4.1), any C-related differences between groups would be expected to be enhanced in males and give clearer understanding of the effects of C in tumour formation and progression. As such, male mice only were used in subsequent experiments to investigate deficiencies in C proteins.

5.2.2. The effect of C1q deficiency on MCA tumour induction in male mice

In order to investigate the role of classical pathway activation, and the multifunctional protein C1q in tumour induction, male mice deficient in this protein, were injected with 3-MCA along with a control group comprising age-matched male WT mice. $C1q^{-/-}$ animals are unable to activate the Ab-dependent arm of C which, as discussed in Chapter 1, has been shown to be a major source of C deposition in several tumour types including renal cell carcinoma (Magyarlaki et al. 1996), breast cancer (Niculescu et al. 1992) and

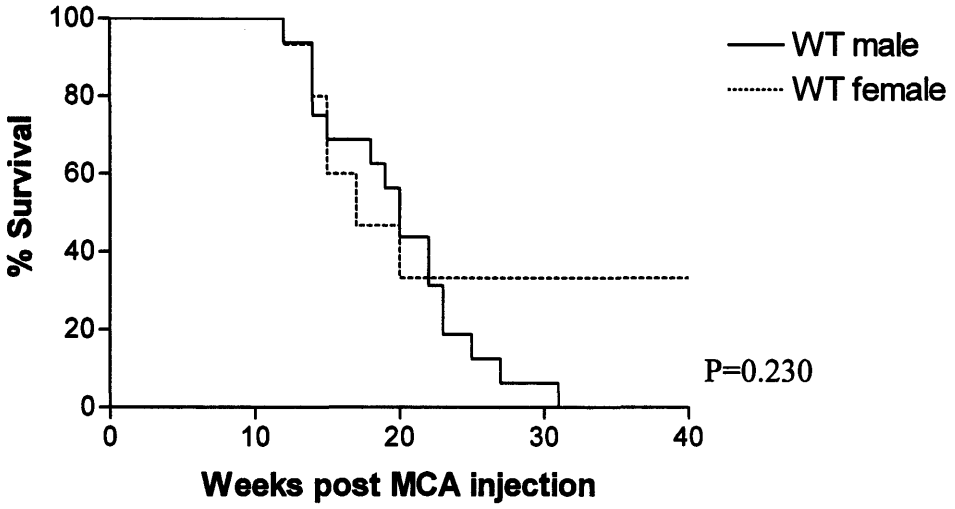


Figure 5.1: 3-MCA induced tumour induction in WT male and female mice.
WT male (n=16) and female (n=16) mice were injected with 400μg MCA, sub-cutaneously into the left hind leg and monitored weekly. Differences between the two groups were analysed using an implementation of the log-rank test. ($p>0.05$)

papillary thyroid carcinomas (Lucas et al. 1996). As such, impaired immune control may be expected in these animals. However, the MBL and alternative pathways are able to function as normal allowing C activation, C3b and MAC deposition. Related effects including opsonisation and anaphylatoxin release (excluding C4a) are also possible through these activation pathways. The amplification capacity of C remains intact, though requires initial deposition of C3b to instigate the amplification.

Data shown in Figure 5.2 (n=24) are compiled from three separate experiments (n=8) performed at different times. As previously, mice were sacrificed when tumours reached 1cm diameter with incidences being noted and pooled. An implementation of the log rank statistical test revealed a statistically significant protective effect in male $C1q^{-/-}$ mice from tumour occurrence ($p=0.002$) compared to WT controls. Notably around 40% $C1q^{-/-}$ mice remained tumour-free at the end of the experiment and tumours arose later in $C1q^{-/-}$ mice than WT counterparts. These data support a role for C1q in promoting tumour growth in WT animals.

5.2.3. The effect of C3 deficiency on MCA tumour induction in male mice

A key experiment in determining the relationship between C and tumour induction involved mice deficient in the central C component C3. These mice are immuno-compromised through impaired B-cell responses and are essentially deficient in C and all of the downstream effects associated with activation. Although the initiation of classical or MBL pathways is possible, lack of C3 will arrest either pathway at an early stage. As such, C-mediated lysis is not expected in these mice, nor any of the wide-ranging effects precipitated by C3 cleavage. As mentioned previously, there are scant data to support C3-independent generation of C5b from C5 (Huber-Lang et al. 2006). However, Figure 4.23 shows evidence of C9 / MAC deposition on tumour cells inoculated into WT but not $C3^{-/-}$ mice, suggesting that C3 is required for initiation of the terminal pathway of C in this model. Whilst the lack of any C-

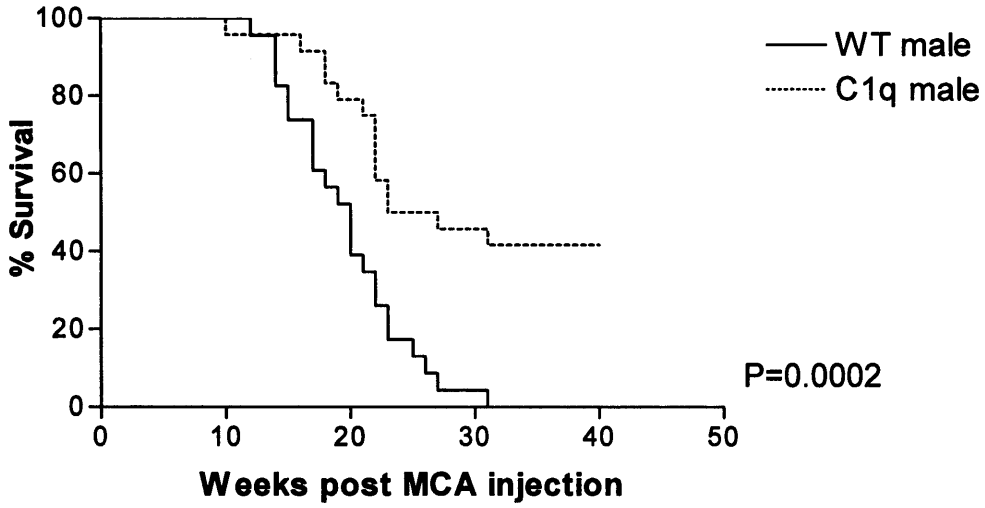


Figure 5.2: 3-MCA induced tumour induction in WT male and C1q$^{-/-}$ mice.
WT (n=24) and C1q knock-out (n=24) male mice were injected with 400µg MCA, sub-cutaneously into the left hind leg and monitored weekly. Differences between the two groups were analysed using an implementation of the log-rank test. ($p<0.05$).

mediated lysis may impact on tumour development, the impairment of associated immune mechanisms including impaired B-cell and T-cell responses may also be of great significance. One such effect is opsonisation, whereby C activation serves to label cells and allow recognition of targets by various immune cell types involved with the anti-tumour response (summarised in Table 1.3). Opsonisation, release of anaphylatoxins (C3a, C5a) or MAC deposition may contribute to observed tumour development and/or progression in WT animals. However, such effects are absent in $C3^{-/-}$ mice.

Data shown in Figure 5.3 (n=24) are compiled from three separate experiments (n=8) performed at different times. Statistical analysis revealed that deficiency in C3 was protective in male mice against MCA-induced tumour growth. As in the case of C1q deficiency, $C3^{-/-}$ mice developed fewer tumours over the course of the experiment with tumours also arising later than in WT controls. The protective effect was found to be statistically significant ($p=0.0017$) and suggests that C3 is promoting tumour progression in WT male mice.

5.2.4. The effect of CD55 and CD59 deficiency on MCA induced tumour induction in male mice

Mice lacking the CRegs, CD55 and CD59, were shown in Chapter 4 to possess a fully functional C system with similar haemolytic capabilities to WT mice (Figure 4.15). However, tumours arising in these animals will lack two potent proteins for controlling or inhibiting C attack suggesting that C activation on tumour cells would be more likely to result in lysis. This impaired control over C deposition may therefore affect tumour induction in these mice.

Figure 5.4 shows that $CD55^{-/-}.CD59^{-/-}$ male mice were more resistant to MCA-induced tumour induction, as compared to age-matched wild type controls (n=24). As previously observed in $C1q^{-/-}$ and $C3^{-/-}$ groups, tumours were slower to arise in the $CD55^{-/-}.CD59^{-/-}$ mice compared to age-matched WT

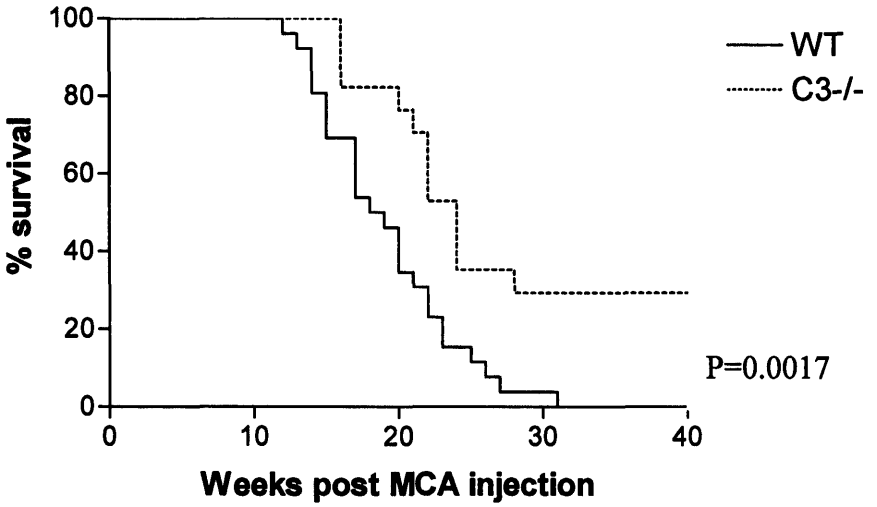


Figure 5.3: 3-MCA induced tumour induction in WT male and $C3^{-/-}$ mice. WT (n=24) and C3 knock-out (n=24) male mice were injected with 400µg MCA, sub-cutaneously into the left hind leg and monitored weekly. Differences between the two groups were analysed using an implementation of the log-rank test. ($p<0.05$).

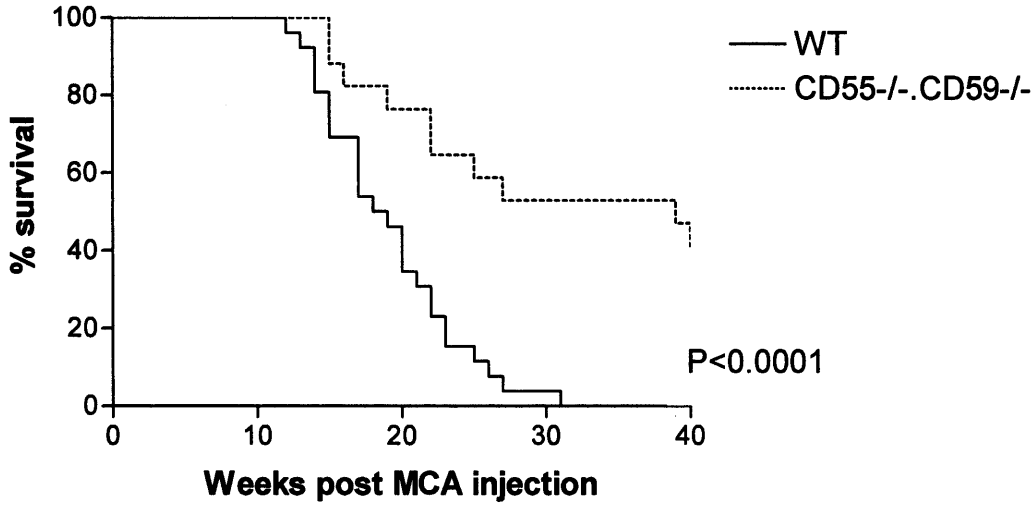


Figure 5.4. 3-MCA induced tumour induction in WT male and CD55$^{-/-}$.CD59$^{-/-}$ mice. WT (n=24) and CD55$^{-/-}$.CD59$^{-/-}$ (n=24) double knock-out male mice were injected with 400µg MCA, sub-cutaneously into the left hind leg and monitored weekly. Differences between the two groups were analysed using an implementation of the log-rank test. ($p<0.05$).

controls. Additionally, a greater proportion of these mice remained free of tumours at the end of the experiment. Statistical analysis of survival data revealed a highly significant difference between the groups ($P<0.0001$). The strong trend demonstrated is for a protective effect of CD55 and/or CD59 deficiency against 3-MCA induced tumourigenesis. These data suggest that one or both of these regulators are involved with tumour progression in WT mice. Whilst this effect could be mediated through C deposition on cells not expressing these CReg (either through CDC or opsonisation), other non-C effects of CReg expression, discussed in detail later, may be contributing (Longhi et al. 2006).

5.3. Post excision characterisation of tumours by flow cytometry

Effective characterisation of tumour cells in an *in vivo* setting can be achieved by flow cytometric staining of tumours immediately post excision. Upon reaching a pre-determined size (1cm diameter), tumours were excised and either frozen for sectioning, or disaggregated and characterised by flow cytometry. CReg expression (CD55, CD59 and Crry) was assessed by this method, and controlled by inclusion of erythrocytes obtained from a WT mouse known to express each of these proteins (Miwa et al. 2002). Additionally, C3b/iC3b deposition was assessed by this method, providing a measure of C activation on the same tumour cell populations.

Cells were disaggregated immediately post-excision, washed three times, strained to obtain a single cell suspension and re-suspended in lysis buffer to remove red blood cells from the preparation. Cells were stained by a two-step staining protocol as detailed in Chapter 2. Cells obtained from dis-aggregated tumours exhibited highly heterogeneous scatter plots, as shown in Figure 5.5. In order to remove cellular debris and select fibrosarcoma population, cells were gated to include those within R1 as shown for fluorescence analysis. Figure 5.6 shows representative histograms from 5 tumours from each group of mice (WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$) stained for CD55, CD59, Crry expression and C3b deposition. As expected, no CD55 or CD59 expression

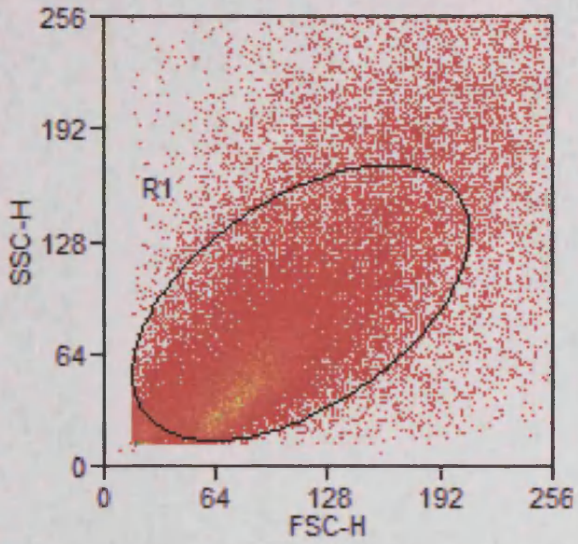


Figure 5.5: Flow cytometric analysis of dis-aggregated fibrosarcoma populations
Flow cytometry data representative of dis-aggregated cells from freshly excised tumour. Heterogeneous tumours were excised, washed, strained and red blood cells lysed. Following antibody staining protocols, cells were analysed using flow cytometry.

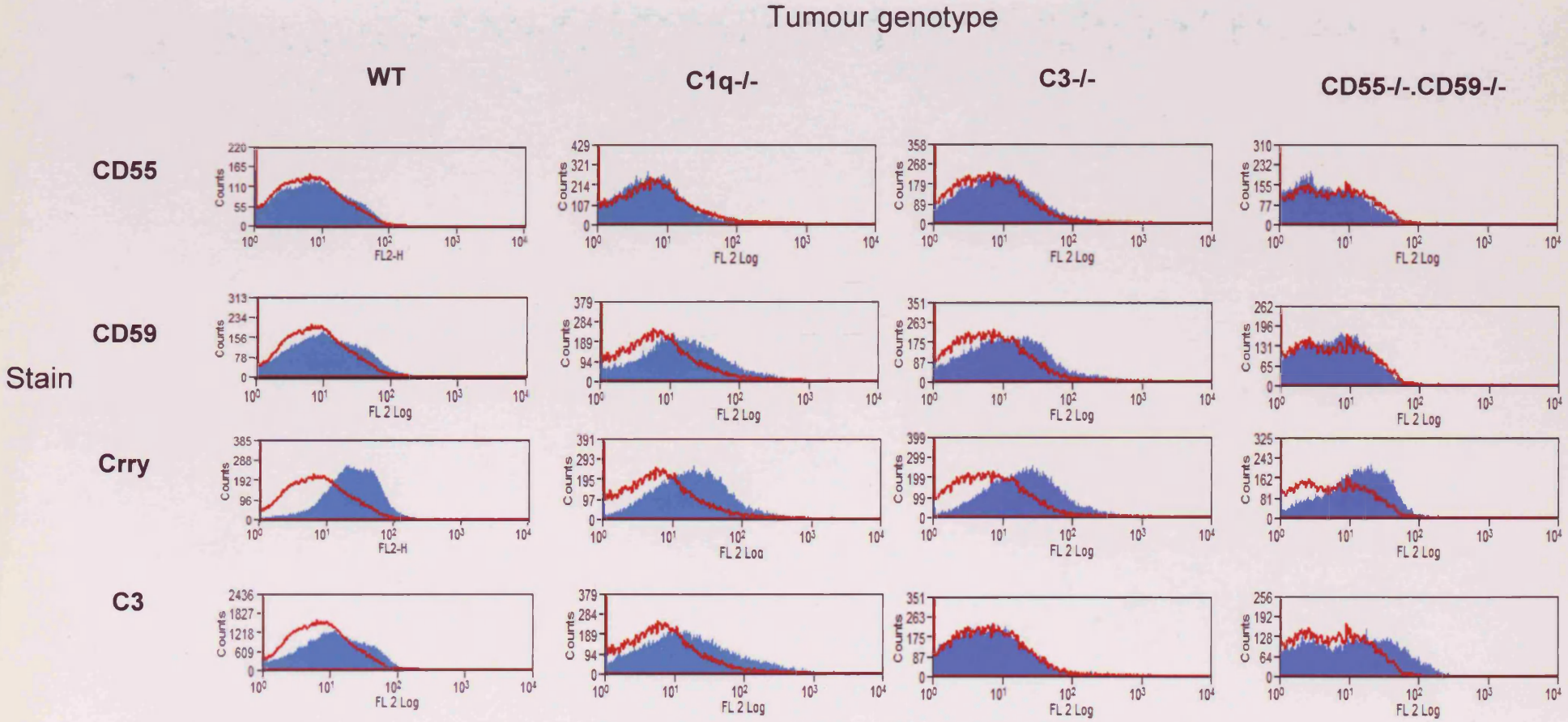


Figure 5.6. Post-excision staining of disaggregated fibrosarcoma cells by flow cytometry.
Cells were washed, strained and re-suspended in red blood cell lysis buffer. Rat anti-mouse CD55 (MD1), rat anti-mouse CD59 (Mel4), rat anti-mouse Crry (5D5) and rat anti-mouse C3 (11H9) were used, followed by detection using RPE-labelled donkey anti-rat IgG. In each case, figure shown is representative of >3 separate experiments. Lines shown in red represent secondary antibody only binding to the same cell population.

was found on cells derived from mice deficient in these CReg, while no C3b was deposited on C3-/- tumour cells. Crry was expressed consistently within each group, while the absence of CD55 was also common to all populations analysed. However, there was some variation in CD59 expression and C3b/iC3b deposition between tumour genotypes. Figure 5.7 shows effective detection of CD55, CD59 and Crry by Mel4, MD1 and 5D5 Abs respectively on mouse erythrocytes confirming that the lack of CD55 observed on fibrosarcoma populations was due to lack of expression and not defective staining.

A potential difference was observed in levels of C3b/iC3b deposited on the tumour cells. Changes in median fluorescence intensity (MFI) compared to control populations are summarised in Figure 5.8 and show WT tumours to exhibit a higher mean level of deposition than was detected in other groups. However, this difference was influenced by one 'flyer' exhibiting particularly high levels of deposition. This tumour population with high levels of C3b/iC3b deposited may represent an anomalous data point yet no statistically significant difference existed between WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ groups. Tumours excised from $C1q^{-/-}$ mice exhibited lower levels of deposition than those from WT hosts suggesting a role for classical pathway activation in C deposition on these cells. However, this observation was found not to be statistically significant when compared to normal (i.e. WT) levels ($p=0.178$). Tumours excised from $CD55^{-/-}.CD59^{-/-}$ mice also showed decreased C3b/iC3b deposition compared to WT controls. However, this effect was not as strong as that observed in $C1q^{-/-}$ tissues and was again found not to be statistically significant ($p=0.195$). The lack of CD55 on these tumours would be expected to lead to enhanced C3b deposition on these cells, however CD55 expression was not detected on any populations analysed in this Chapter and was not required for progression of these tumours *in vivo*. Comparison of C3 fragment deposition on WT and $C3^{-/-}$ cells was found to be statistically significant ($p=0.022$).

Figure 5.9 shows summarised data whereby changes in median MFI observed following staining for CD59 are plotted for each group (n=5). The

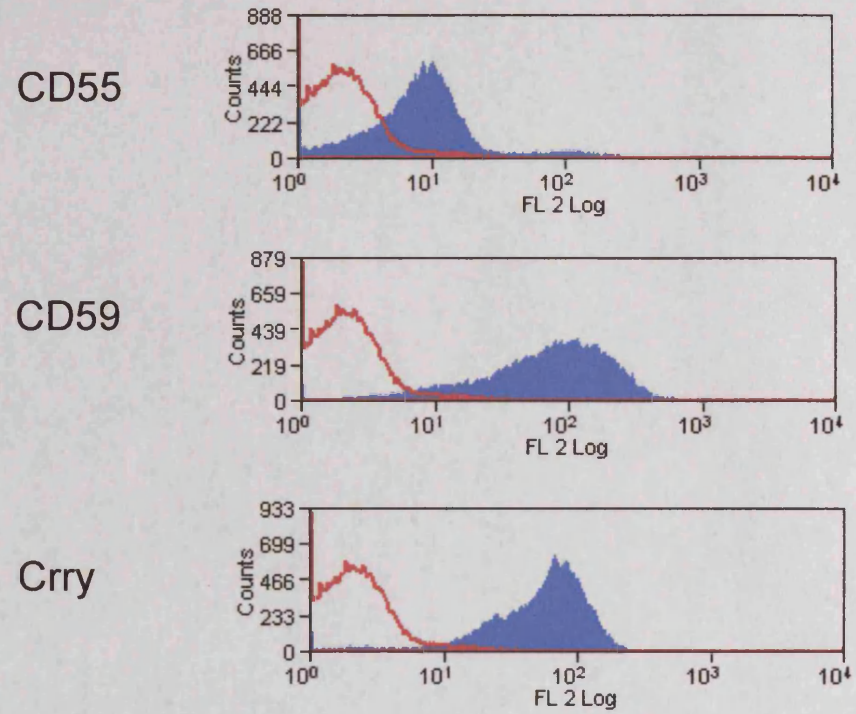
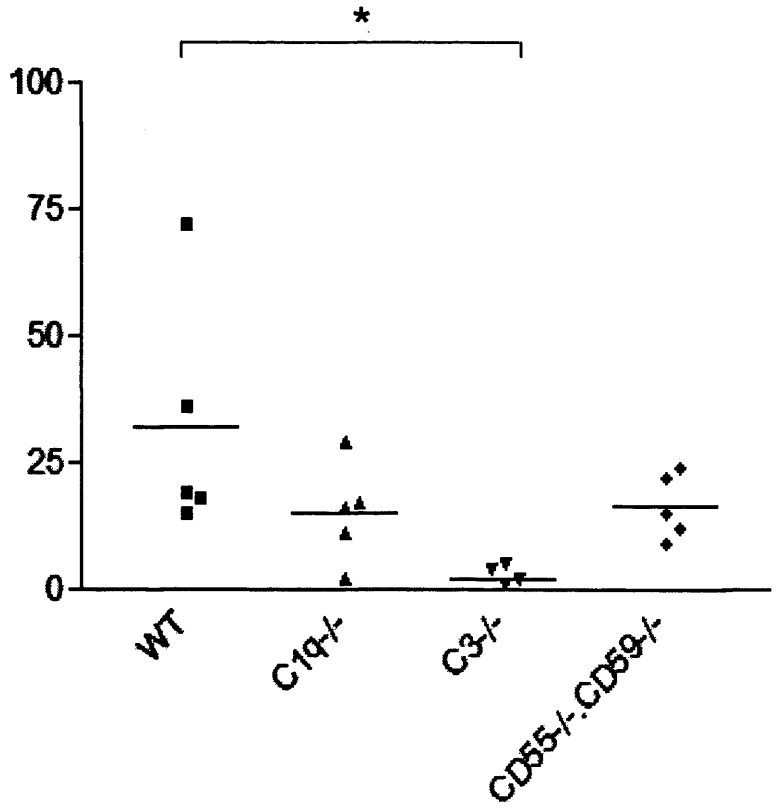
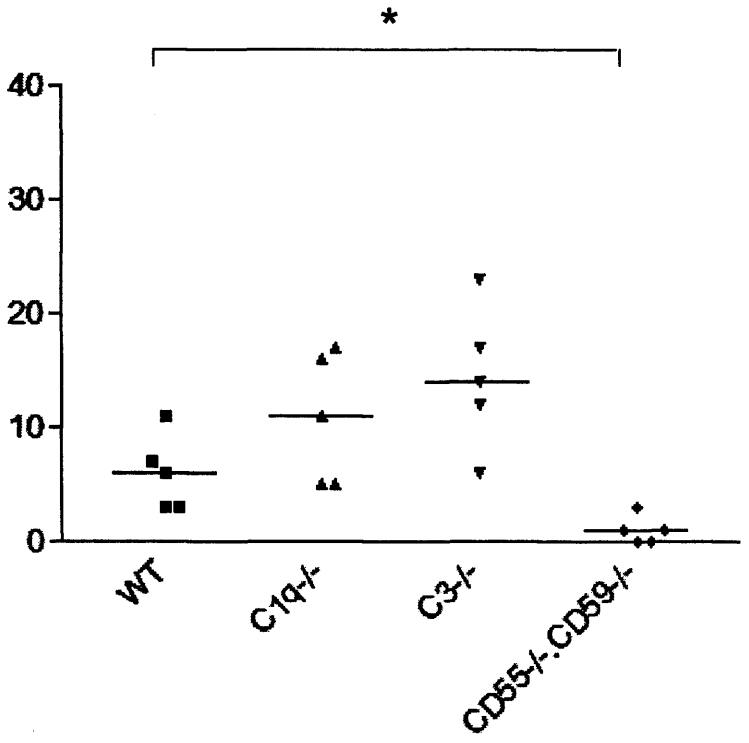


Figure 5.7: CReg staining on mouse erythrocytes
Staining of mouse erythrocyte cells by flow cytometry. Cells were washed and incubated with rat anti-mouse CD55 (MD1), rat anti-mouse CD59 (Mel4), rat anti-mouse Crry (5D5) followed by detection using RPE-labelled donkey anti-rat IgG. In each case, figure shown is representative of >3 separate experiments. Mouse erythrocytes were used as a positive control for presence of CReg. Lines shown in red represent secondary antibody only binding to the same cell population.



	Mean ± SEM
WT	32 ± 10.65
$C1q^{-/-}$	15 ± 4.39
$C3^{-/-}$	1.8 ± 1.39
$CD55^{-/-}.CD59^{-/-}$	16.4 ± 2.87

Figure 5.8: Flow cytometric analysis of C3b/iC3b deposition
Changes in median fluorescent intensity following C3 staining were recorded and plotted. Data were analysed using a 2 tailed, unpaired t-test. * indicates $p<0.05$.



	Mean ± SEM
WT	6 ± 1.48
$C1q^{-/-}$	10.8 ± 2.58
$C3^{-/-}$	13.2 ± 3.24
$CD55^{-/-}.CD59^{-/-}$	1 ± 0.55

Figure 5.9: Flow cytometric analysis of CD59 expression

Changes in median fluorescent intensity following CD59 staining were recorded and plotted (n=5). Data were analysed using a 2 tailed, unpaired t-test.

majority of tumours were found to express low levels of CD59 with the median expression higher in $C1q^{-/-}$ and $C3^{-/-}$ tumours. When compared to WT, differences between $C1q^{-/-}$ ($p=0.145$) and $C3^{-/-}$ (0.080) groups were not statistically significant. However, the observation that tumour cells express CD59 is relevant and provides a mechanism for inhibition of lysis by regulation of the terminal pathway. The observed difference in CD59 expression between WT and $CD55^{-/-}.CD59^{-/-}$ tumour populations was statistically significant ($p=0.013$).

5.4. Immunohistochemical staining of MCA-derived tumours

5.4.1. Morphology

Excised tumours showed expected morphology for fibrosarcomas induced via 3-MCA induction, as previously published (Blankenstein and Qin, 2002). Figure 5.10 shows tumours to exhibit high density of cells, with haematoxylin and DAPI nuclear stains particularly prominent.

5.4.2. C3 staining

Once excised, tumours were frozen, sectioned and stained using histochemical techniques as described in Chapter 2. A rat mAb against mouse C3 was used to visualise deposition of C3. This antibody is specific for C3, C3b and iC3b (Mastellos et al. 2004), and provides a valuable indicator of C3b or the associated inactivation product iC3b deposited on membranes.

Significant C3b/iC3b deposition was observed in all tumour sections analysed (Figure 5.11), excepting those derived from animals deficient in C3 where no fluorescent signal was observed. It is not possible to determine from these data the source(s) of the C3 products. These may be tumour derived, a product of tumour cells activating host C or a combination of both mechanisms. Control tissues treated with isotype control mAbs and secondary Abs all exhibited no fluorescence. Thus, staining with the anti-C3 primary Ab was specific.

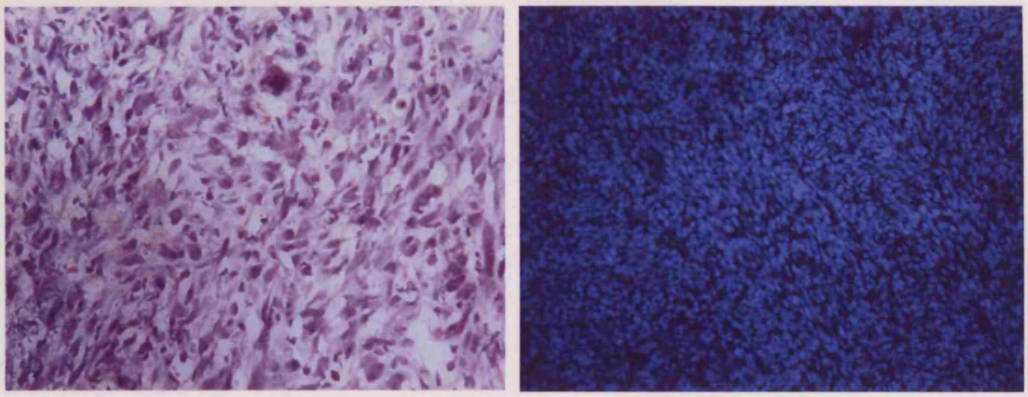


Figure 5.10: Nuclear staining of 3-MCA induced tumours
3-MCA induced tumours were excised and stained with haematoxylin (left panel) or 4',6-diamidino-2-phenylindole (DAPI) (right panel) in order to localise fibrosarcoma nuclei. Data shown are representative slides of fibrosarcomas excised from WT animals.

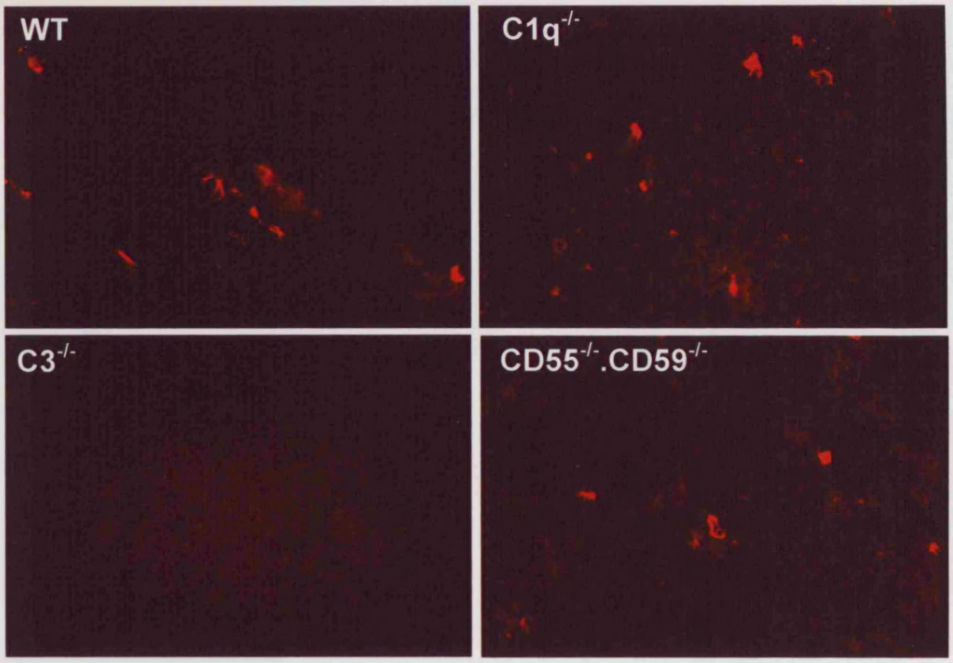
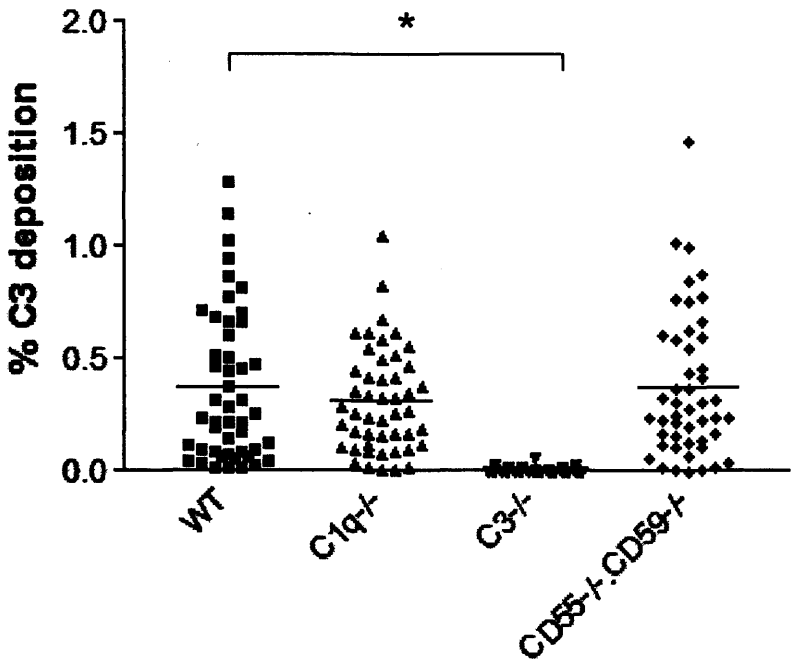


Figure 5.11: C3b/iC3b deposition on 3-MCA induced tumour sections
C3 cleavage and deposition on MCA induced tumours was assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of diseased tissue (not shown) using a rat monoclonal anti-mouse C3/C3b/iC3b antibody. Alexa-fluor 594 conjugated donkey anti-rat secondary antibody was used to detect. Images shown are representative sections from each tumour (n=6). Tissue from each genotype of tumour was also stained using an isotype control antibody, negative staining was found.

In order to quantify C3b/iC3b deposition on tumour sections, and identify any differences between tumour genotypes, sections were analysed using Image Pro Analyser software. Use of this software allows a fluorescence threshold to be set, above which any signal is marked positive. The percent area stained positive can then be calculated per random camera frame. Eight frames per tumour were analysed, with 6 tumours assessed per genotype. These data are summarised in Figure 5.12 and show comparable levels of deposition in each group, with the exception of $C3^{-/-}$ animals where no positive signal was observed. Statistical analysis revealed no differences in deposition between WT and $C1q^{-/-}$ ($p=0.320$) or $CD55^{-/-}.CD59^{-/-}$($p=0.990$) tumour sections. As expected, a strong statistically significant difference was observed between WT and $C3^{-/-}$ sections ($p<0.001$). This provides a useful negative control and confirms the specificity of the Ab and staining procedure used.

5.4.3. C9 staining

Deposition of C9 was assessed to determine the presence of MAC on tumour tissue sections. An affinity purified Ab against rat C9 was used and fluorescence quantified as described above. As shown in Figure 5.13, significant C9 deposition was observed in tumour sections excised from WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice. No C9 deposition was observed on sections from $C3^{-/-}$ mice suggesting that C3 is required for initiation of the terminal pathway. Collated data following quantitation of fluorescence levels are shown in Figure 5.14 and reveal comparable levels of C9 between WT and $C1q^{-/-}$ ($p=0.897$), and $CD55^{-/-}.CD59^{-/-}$ ($p=0.923$) groups. As expected, a strong significant difference was observed between WT and $C3^{-/-}$ groups ($p<0.001$). Negligible fluorescence was observed on $C3^{-/-}$ tumour cells indicating C3-dependent terminal pathway activation in WT and suggests that tumour cells in this model do not secrete and deposit C9 on their own surfaces.



	Mean ± SEM
WT	0.371 ± 0.049
$C1q^{-/-}$	0.312 ± 0.033
$C3^{-/-}$	0.003 ± 0.001
$CD55^{-/-}.CD59^{-/-}$	0.372 ± 0.047

Figure 5.12: Immunohistochemical analysis of C3b/iC3b deposition
Percentage area stained positive for C3 deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test. * represents $p<0.05$.

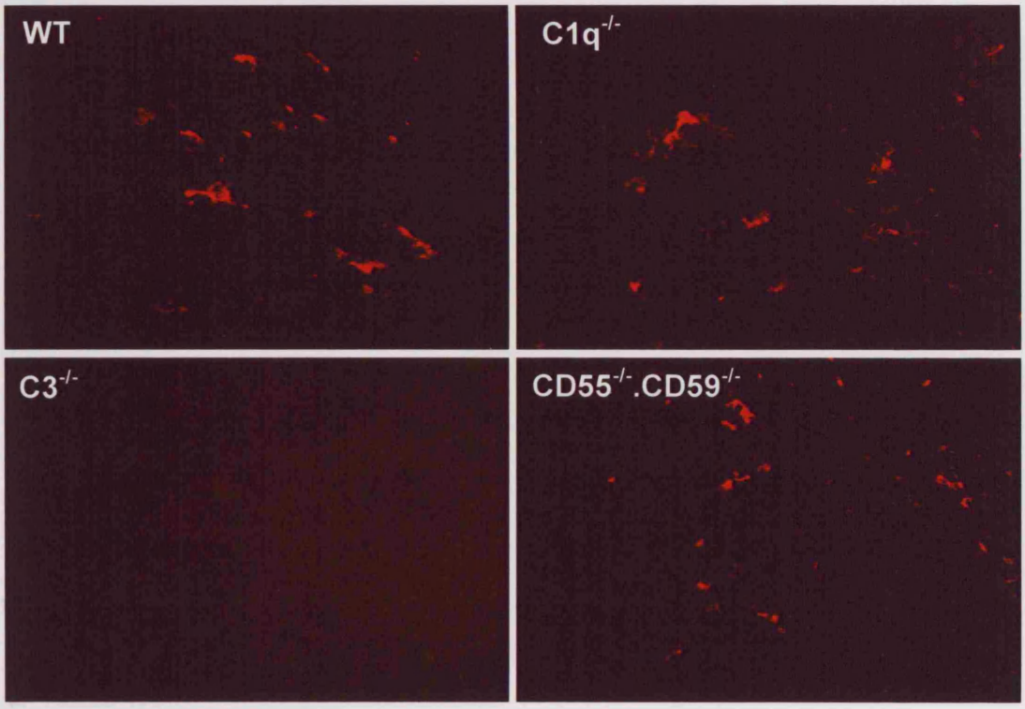
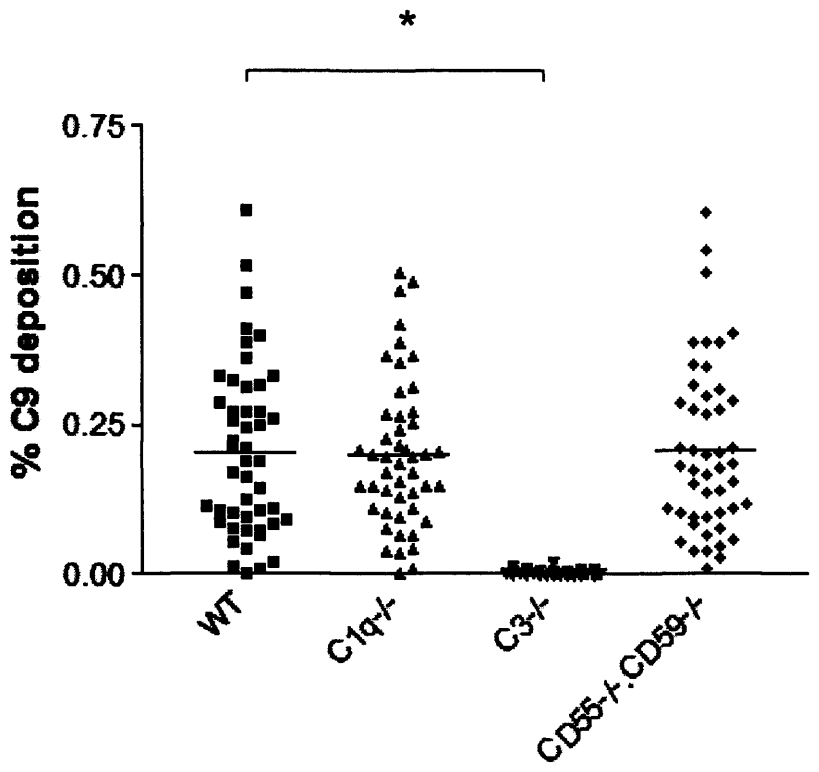


Figure 5.13: C9 deposition on 3-MCA induced tumour sections
C3 cleavage and deposition on MCA induced tumours was assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of diseased tissue (not shown) using a rabbit anti-rat C9 antibody. Alexa-fluor 594 conjugated goat anti-rabbit secondary antibody was used to detect. Images shown are representative sections from each tumour (n=6).



	Mean ± SEM
WT	0.201 ± 0.021
$C1q^{-/-}$	0.202 ± 0.018
$C3^{-/-}$	0.004 ± 0.001
$CD55^{-/-}.CD59^{-/-}$	0.372 ± 0.047

Figure 5.14: Immunohistochemical analysis of C9 deposition
Percentage area stained positive for C9 deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test. * represents $p<0.05$.

5.4.4. CD4 staining

It has previously been reported that CD4+ T cell responses are enhanced in mice lacking CD59 (Longhi et al. 2005). Thus it is possible that tumours are better controlled in mice lacking this Creg as a consequence of more robust anti-tumour CD4+ T cell activity. Infiltration of CD4+ cells, the majority of which are T cells (Awen Gallimore, personal communication), into MCA-induced tumours was assessed by immunohistochemistry. This protein is a widely used marker for T-lymphocytes and was observed in all tumour tissues analysed as a defined ring of fluorescence (Figure 5.15). In order to assess differences between groups, numbers of infiltrating CD4+ cells were counted (8 frames per tumour, n=6, Figure 5.16). No observable differences were found between groups, with a mean of ~5 CD4+ cells found per tumour section. These data indicate no difference in T-cell accumulation in tumour development in $C1q^{-/-}$, $C3^{-/-}$ or $CD55^{-/-}.CD59^{-/-}$ or WT hosts.

5.5. Discussion

The 3-MCA tumour induction model provides powerful reproducible data on the relative susceptibilities of WT and C component/CReg deficient mice to 3-MCA induced fibrosarcomas. Wild type mice were found to be more susceptible to tumour development than $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice. In each case, the protective effect of C component/CReg deficiency was found to be statistically significant.

Induction of 3-MCA tumours in WT mice was observed with high incidence and found to be consistent with previous studies with the majority of tumours arising 15-25 weeks post 3-MCA administration (Figure 5.1). Differences in tumour induction observed in male and female WT mice were not statistically significant ($p=0.23$). However, while all 16 male mice developed a tumour by week 31, 5 from 16 female mice remained tumour free throughout the experiment. Sarcomas have previously been shown to elicit a greater immune

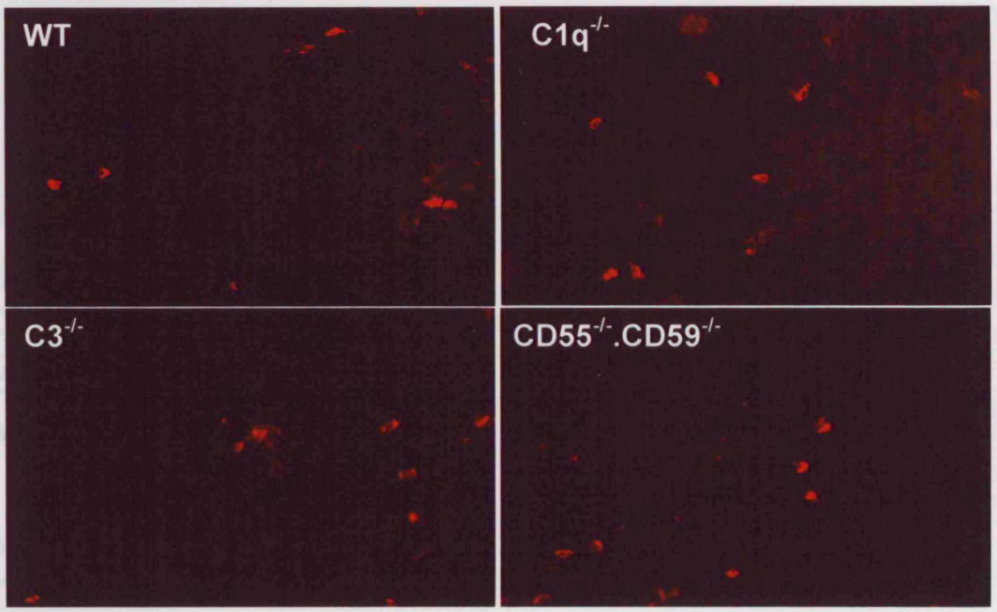


Figure 5.15: CD4+ Cell infiltrate in 3-MCA induced tumour sections
CD4+ cells in MCA induced tumours were assessed by a two step staining protocol as detailed in Chapter 2. Staining was optimised on sections of spleen (not shown) using a rat monoclonal anti-mouse CD4 antibody. Alexa-fluor 594 conjugated donkey anti-rat secondary antibody was used to detect. Images shown are representative sections from each tumour (n=6). Tissue from each genotype of tumour was also stained using an isotype control antibody, negative staining was found.

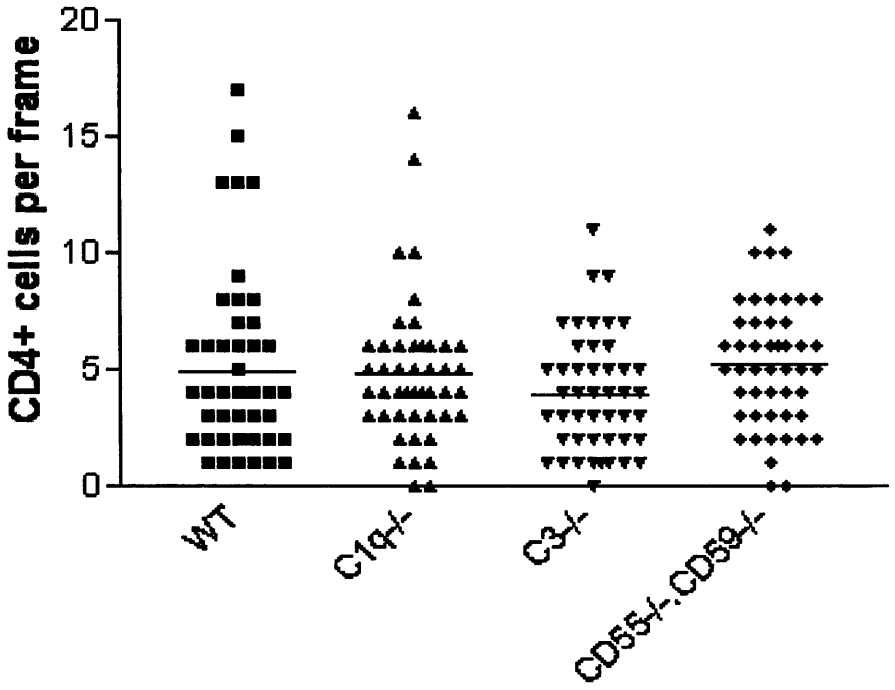


Figure 5.16: Immunohistochemical analysis of CD4+ infiltrate
Numbers of CD4+ cells per section were counted, 8 frames were assessed per tumour with 6 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test.

response in female mice than males (Pollack 1971), though many other factors can contribute to 3-MCA tumour induction e.g. age, sex, strain and environmental conditions. As shown in Chapter 4 (Figure 4.1), male mice possess significantly higher C activity than females. The stronger C response in male mice may contribute to the higher tumour incidence observed although numerous other factors, most notably those linked to 3-MCA metabolism, are likely to be involved with differing enzyme levels thought to be important (Salerno, Ramm and Whitmire 1973). Various enzymes are required to metabolise 3-MCA into genotoxic forms and allow carcinogenesis with cytochrome P450 enzymes being of particular importance (Moorthy et al. 2002). Gender differences in expression of these enzymes have been reported, largely dependent on growth hormones (Sharma et al. 1998). In stark contrast to observations in rats and humans, female mice are consistently found to express higher levels of cytochrome enzymes (Salerno et al. 1973, Pampori and Shapiro 1999, Jarukamjorn et al. 2006). However, gender differences are much more subtle in these models with 40-100% increases typical compared to 300-500% observed in rats. Given these gender differences, the higher tumour incidence indicated in Figure 5.1 and the stronger C system in male mice, further experiments in WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice were performed using male animals only.

Mice lacking C1q are unable to activate the classical pathway of C, shown previously to be a major source of C targeted to tumour cell surfaces (Lucas et al. 1996, Niculescu et al. 2002). Activation and deposition of C in these animals must therefore occur via the alternative or MBL pathway. The hypothesis that the C-system may attack arising tumour cells through the classical pathway leading to tumour elimination or control was tested. However, data obtained in $C1q^{-/-}$ mice showed fewer tumours arising, and at a later date implying that C may instead be promoting tumour development. These data support other emerging data in the field, that events occurring in the C-sufficient mice could be enhancing tumour cell survival and driving proliferation. It seems likely that C1q deficiency demonstrates a protective effect through inability to initially deposit C3, curtailing the amplification

capacity and denying these tumour cells the proliferative stimuli detailed in Chapter 1. However, C1q has numerous roles outside of C activation, which may contribute to the observed pro-tumour effect. A potential mechanism for this is a role for C1q and other classical pathway components in clearance of apoptotic cells (Taylor et al. 2000), which if impaired could result in multiple apoptotic bodies – a known feature of $C1q^{-/-}$ mice (Botto et al. 1998). This in turn could lead to enhanced alternative pathway activation (Matsui et al. 1994) and the proliferative effects associated with C deposition. A wealth of evidence exists to support a role for C1q in binding apoptotic blebs and aiding phagocytotic clearance of these cells. C1q is known to bind directly to blebs and induce C activation (Nauta et al. 2002). However in the absence of C1q, the alternative pathway of C can also be activated (Matsui et al. 1994, Tsuji, Kaji and Nagasawa 1994). Opsonisation of apoptotic cells in this way has been shown to label them for phagocytosis by macrophages (Mevorach et al. 1998, Ogden et al. 2001) and clearance from tissues. Impaired clearance of apoptotic cells in $C1q^{-/-}$ animals may lead to accumulation of dying cells within the tumours of these mice. These apoptotic cells may then be stimulating the alternative pathway and leading to increased C3 deposition. This effect could be responsible for making up any shortfall caused by a lack of classical pathway activation. This would mean that C3 fragments deposited on $C1q^{-/-}$ tissues are not a marker for C activation by progressively growing tumour cells and that C is not deposited on actively proliferating cells. Flow cytometric data showed marginally higher levels of C3b/iC3b deposition on WT tumour cells. However, the mean shift in fluorescence was skewed in the WT group by one population on which C3b/iC3b staining was much stronger. Nevertheless, the differences between WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ groups were shown not to be significant. A statistically significant difference was only observed between WT and $C3^{-/-}$ groups. Further analyses by immunohistochemistry support this observation and found comparable levels of both C3b/iC3b and C9 deposition on WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ tumour sections. As found by flow cytometry, a statistically significant difference was found between WT and $C3^{-/-}$ groups. Whilst the data in Figure 5.2 suggest a pro-tumour role for C1q, it is unclear whether this is mediated through C

activation given the comparable levels of C3b/iC3b and C9 deposition observed via flow cytometry and immunohistochemistry.

Differences in tumour induction in $C3^{-/-}$ mice were also shown to be statistically significant compared to WT controls. The observed difference was again a protective effect against tumour development and progression conferred by a deficiency in C. These data support the findings in $C1q^{-/-}$ mice and point to a role for C in driving tumour growth in WT mice. Tumours arising in these mice may activate C via CP or MBL pathway but have no capacity for C3b deposition and therefore all downstream effects of C activation are abrogated. In these animals there can only be release of C4a anaphylatoxin and not the more biologically active C3a and C5a fragments. The strength of the protective effect ($p=0.0017$) is also important and suggests that C3 may play a major role in promoting tumour progression in WT mice. $C3^{-/-}$ mice essentially possess a non-functional C system and as such are severely immuno-compromised with no lytic capacity and markedly impaired opsonisation. As discussed in Chapter 1, C3b and associated breakdown products have numerous important immune effects outside of C activation including B-cell responses (Rickert 2005), T-cell activation (Knopf et al. 2008), dendritic cell differentiation (Reis et al. 2008) and NK cell activation (Wang et al. 2008). The observation that it was considerably more difficult to induce tumours in these mice point to a pro-tumour role for C3 even with the impairment of several immune mechanisms known to contribute to immunosurveillance. As with $C1q^{-/-}$ mice, these mice exhibit impaired clearance of apoptotic cells through inability to deposit C3 activation products (Taylor et al. 2000, Morelli et al. 2003). However, in contrast to $C1q^{-/-}$ mice, a build of apoptotic blebs would not activate the C alternative pathway in $C3^{-/-}$ hosts. The deposition of neither C3b/iC3b nor C9 was detected on tissue from $C3^{-/-}$ mice either by flow cytometry or immunohistochemical staining. The absence of deposited C3 products on these tissues indicates that $C3^{-/-}$ tumour cells are not subjected to any of the stimulatory effects mediated by deposition of these proteins, which may be responsible for the reduced tumour induction in these mice. A role for C3 activation and deposition has been demonstrated in increased cellular proliferation by normal and

transformed cells (Kuraya et al. 1990). Further, such deposition is known to induce proliferation of a mouse carcinoma line (di Renzo et al. 1999) later shown to be mediated through protein kinase activation (Longo et al. 2005). Numerous effects associated with MAC deposition have been described (see section 1.12) and are also absent in tumours generated in $C3^{-/-}$ mice.

Impaired tumour incidence and progression were also observed in $CD55^{-/-}$.$CD59^{-/-}$ male mice compared to WT controls. This observation cannot be explained by a pro-tumoural role for C. $CD55^{-/-}$.$CD59^{-/-}$ mice have a fully functional C system, allowing activation of all three pathways. This is evidenced by the comparable haemolytic capabilities observed in these and WT mice (Figure 4.15). Induction of tumours in these mice gives rise to tumour cell populations unable to synthesise and express CD55 or CD59. The lack of these proteins may lead to increased C-susceptibility through impaired regulation of both activation pathways (CD55) and the terminal pathway (CD59). Therefore the decreased tumour incidence, and slower growth rates observed in these animals may be attributable to enhanced clearance of tumour cells via CDC. However, no tumours examined in this model *in vivo* or *in vitro* were shown to express CD55 suggesting that expression of this regulator on tumour cells does not normally influence progression. Expression of CD59 was detected on excised tumours from WT, $C1q^{-/-}$ and $C3^{-/-}$ mice, though at relatively low levels. This protein is a potent inhibitor of MAC formation and may play a role in protecting 3-MCA induced tumours against C attack thus contributing to the higher incidence and faster growth rate of tumours induced in WT compared to $CD55^{-/-}$.$CD59^{-/-}$ mice. This protein has previously been associated with enhanced tumour growth and is considered a promising therapeutic target (Macor et al. 2007, Donev et al. 2008). The observation that comparable amounts of C3b/iC3b and C9 were deposited on tumours arising in $CD55^{-/-}$.$CD59^{-/-}$ and WT mice (found by flow cytometry and immunohistochemical techniques) is expected given the similar lytic capabilities of these mice and suggests that tumour populations in these mice activate C to similar extents.

CD59 expression was detected on tumour cells derived from WT, $C1q^{-/-}$ and $C3^{-/-}$ mice with no statistically significant difference observed despite a trend for elevated levels in tumours from $C3^{-/-}$ animals. The presence of CD59 and absence of CD55 in these tissues suggests that CD59 expressed on tumour cells may contribute to tumour progression. The role of CD59 is primarily to regulate the terminal pathway by preventing the incorporation of C9 molecules into the cell membrane. As such, elevated levels of C9 deposition would be expected in mice lacking CD59 compared to WT and $C1q^{-/-}$ mice which have been shown to deposit significant amounts of C3b/iC3b and express CD59. However, data shown in Figure 5.14 do not support this and indicate comparable levels of C9 deposition between groups with no elevated deposition observed on $CD55^{-/-}.CD59^{-/-}$ tumour tissue. Further investigation into the individual effects of CD55 and CD59 deficiency would be achieved using mice deficient in CD59 only.

A crucial consideration in this study is that differences in tumour growth may be independent of CD55 or CD59 expression by the tumour cells themselves. Expression of these regulators has recently been shown to have important effects on APCs and/or T cells in both $CD55^{-/-}$ and $CD59^{-/-}$ mice. Mice lacking CD55 have heightened T cell responses, measured by proliferation and IFNγ production, compared to controls with normal CD55 expression (Liu et al. 2005, Heeger et al. 2005). This effect, which was accompanied by a decrease in IL-10 production, was attributed to lack of CD55 on T-cells in one study (Liu et al, 2005). However, the parallel study found CD55 expression on both T-cells and dendritic cells to play a role, with enhanced C activation on the APCs necessary and C3, C5 and fD demonstrated to contribute to enhanced responses (Heeger et al 2005). Additionally, CD4+ T-cells have been shown to proliferate more extensively in response to antigen in $CD59^{-/-}$ mice compared to WT controls (Longhi et al. 2005). This effect, unlike effects mediated by CD55, was shown to be independent of C activation. Thus, reduced tumour incidence observed in $CD55^{-/-}.CD59^{-/-}$ mice could reflect stronger anti-tumour T-cell responses in these mice compared to WT controls.

Initial analysis of CD4+ cellular infiltrate in tumour sections revealed no differences between WT and $CD55^{-/-}.CD59^{-/-}$ tissues with infiltrating cells readily observed in both. Whilst this may imply no role for CD4+ T cells in promoting tumour rejection, effects may be due to different T-cell activities, rather than recruitment or proliferation of these cells. It is also possible that at this stage in tumour development, progressing tumours have evaded the immune responses rendering the T-cells ineffective. Analysis of anti-tumour T-cell responses in tumour-free WT and $CD55^{-/-}.CD59^{-/-}$ mice may therefore prove more informative in elucidating their influence on tumour progression.

Data described in this Chapter reveal clear differences between 3-MCA induced tumour growth in WT mice and those deficient in the C components C1q and C3. A fully functional C system appeared to have an important pro-tumour role in this model and was able to drive tumour progression in WT mice. Mice with impaired C responses developed tumours at a slower rate with a number of mice remaining tumour free at the end of the experiment. Additionally, mice deficient for CD55 and CD59 were also found to be resistant to tumour induction. Whilst tumours lacking CReg would be expected to be more prone to clearance, the impact of these CReg on T-cell activity may also be important. Complement activation and deposition was observed in 3-MCA induced tumours, though it is not clear whether C3 fragments are derived from host C activation, or synthesis by tumour cells. In order to address this question, novel cell lines were generated from tumours excised from the C-deficient and WT mice. Generation and use of these lines is detailed in the following Chapter.

6. Generation and characterisation of new fibrosarcoma lines from MCA-induced tumours

6.1. Introduction

The aims of the experiments described in this chapter were to generate, select and characterise fibrosarcoma cell lines of known C genotypes. Tumours were induced by 3-MCA carcinogenesis in WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice, excised and cultured *in vitro*. These heterogeneous fibrosarcoma populations possess the same genotype as the mouse in which they were raised and can be examined *in* vitro to identify behavioural or phenotypic differences associated with each deficiency listed above. The following characteristics are of particular relevance to this study and were assessed in this Chapter: fibrosarcoma phenotype, *in vitro* proliferation rate, CReg expression, C3 secretion, *in vitro* sensitivity to complement and *in vivo* growth in WT and $C3^{-/-}$ hosts.

Due to the multi-cellular origin of 3-MCA induced tumour populations (Reddy and Fialkow 1979), clonal cell lines were also generated and expanded from the original populations. These novel lines maintain the genotype of the parent line and are derived from one cell thus providing a more stable and uniform model for interpretation of the above characteristics. An additional advantage to this technique is that clones (of differing genotypes) exhibiting comparable *in vitro* characteristics can be selected allowing intra-experimental control for subsequent *in vivo* experiments.

Differences observed between heterogeneous populations or clones of different genotypes may provide interesting insights into the respective roles of individual C components in tumour cell phenotype. Additionally, re-inoculation of lines sufficient and deficient in C3 allows investigation of the source of this protein observed by immunohistochemistry on the tumour cell surfaces. A typical experiment as outlined in Table 6.1 would indicate whether activated C3 is derived from the host animal or secreted by the tumour cells as has been reported and described in Section 1.13.

Tumour Line	Recipient Animal	Complement Source
C3 -	$C3^{-/-}$	No C3
C3 +	$C3^{-/-}$	Local (tumour C3 only)
C3 -	$C3^{+/+}$	Systemic C3
C3 +	$C3^{+/+}$	Local & Systemic C3

Table 6.1: Control over host/tumour derived C3 secretion
Mice sufficient and deficient in C3 are injected with fibrosarcoma cells with and without the ability to synthesise C3. In this way, the effects of systemic and tumour-derived C3 on fibrosarcoma incidence or growth can be assessed.

6.2. Cloning of fibrosarcoma lines

Induction of fibrosarcomas by 3-MCA gives rise to highly heterogeneous tumour populations of multi-cellular origin. As such, clonal lines offer a more homogeneous and consistent phenotype. For this reason, a number of clones (n=8) were isolated from each genotype of tumour cells collected. WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ tumours were excised, disaggregated, washed and cultured as described in Chapter 2. Many cells did not survive, and a number of non-fibrosarcoma cells were also present in the initial culture populations. These cells were quickly outgrown and fibrosarcoma cells rapidly became the dominant cell type in culture samples. Once a healthy fibrosarcoma population was observed, clonal lines were isolated as described in Chapter 2. Briefly, cells were plated at <1 cell per well and wells containing only one clone were selected, lifted with an EDTA solution, and grown out. This procedure was repeated 3 times as described in section 2.3.3. Figure 6.1 shows an example of flow cytometry data with cells from each tumour genotype stained with a fibroblast marker (anti-CD140a). This Ab binds to Platelet Derived Growth Factor Receptor-alpha (PDGFRα) on cell surfaces and was used to confirm a fibroblast phenotype. Populations were stained prior to and following the cloning procedure. Initial heterogeneous populations (column A) showed a broad range of CD140a expression, while clones displayed a more homogeneous histogram with vastly reduced variation

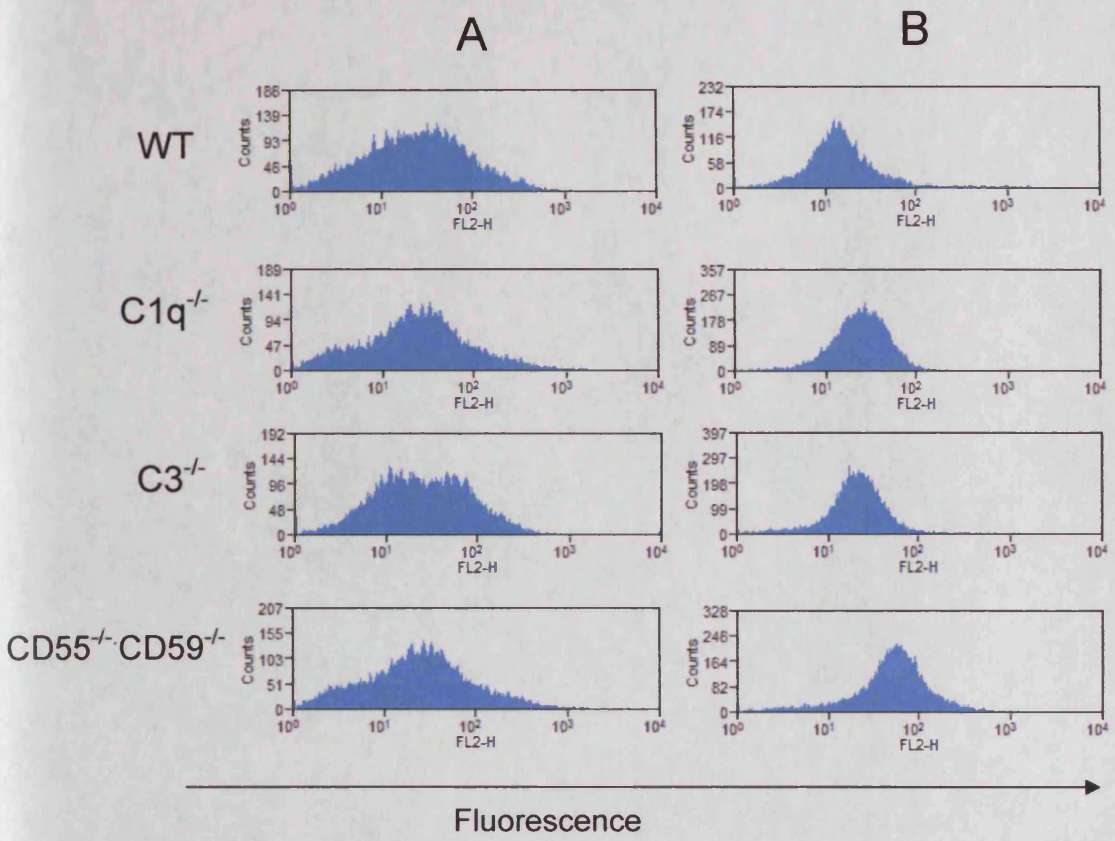


Figure 6.1: CD140a expression on parent and clonal fibrosarcoma lines
Cultured cells from excised tumours were stained by flow cytometry for expression of CD140a pre and post cloning. Apa5 (rat anti-mouse CD140a) was visualised using donkey anti-rat IgG-RPE. Column A depicts one representative cell population from each genotype after culturing. Column B depicts final population after 3 cloning steps.

(column B). Expression of CD140a was variable and appeared relatively low-level. This observation may be attributable to low CD140a expression on these cells, or a poor affinity Ab. However, it has been previously shown that tumours derived through 3-MCA carcinogenesis often down-regulate fibroblast-specific markers (Kopp, Croci and Trueb 1995, Schreier et al. 1988). This effect may also be affecting CD140a detected on these cells. CD140a was detected in all fibrosarcoma populations (both pre- and post-cloning) and provided a useful indicator of hetero/homogeneity of populations. At least 8 clonal lines of each genotype were selected and cultured.

6.3. *in vitro* comparison of clonal proliferation rates

The use of 3-MCA induced carcinogenesis results in a multitude of random mutagenic events. This and the heterogeneity of resulting populations suggest that differences are likely to be observed between clones of the same genotype. In order to identify and select clones with equivalent proliferative rates, an *in vitro* assay was optimised utilising a colorimetric method based on metabolism. The method is detailed in Chapter 2 and involves incubation of a known number of cells with a non-toxic coloured metabolite (10% Alamar Blue™) on a daily basis. The rate of metabolite consumption is proportional to the number of cells per well and a quantifiable colour change is observed upon metabolism of the compound. The colour change was observed as a result of a reduction-oxidation (REDOX) indicator dye included to detect reduction of the Alamar Blue compound. Observed colour change over a 2 hour period was quantified via fluorescence measurement and population expansion plotted over a 10 day period. Cells were plated at $1.5x10^4$ per well in triplicate and assayed on 10 consecutive days.

Figure 6.2 shows proliferation of 8 separate WT clones as measured over a 10 day period. As expected, there was some variation between the clones though all followed a sigmoidal curve pattern, characterised by a lag, log and stationary phase where all the metabolite was consumed. After 10 days, data became erratic as cell death occurred through lack of space and nutrients. Two clones grew noticeably slower than the majority, while another grew at a

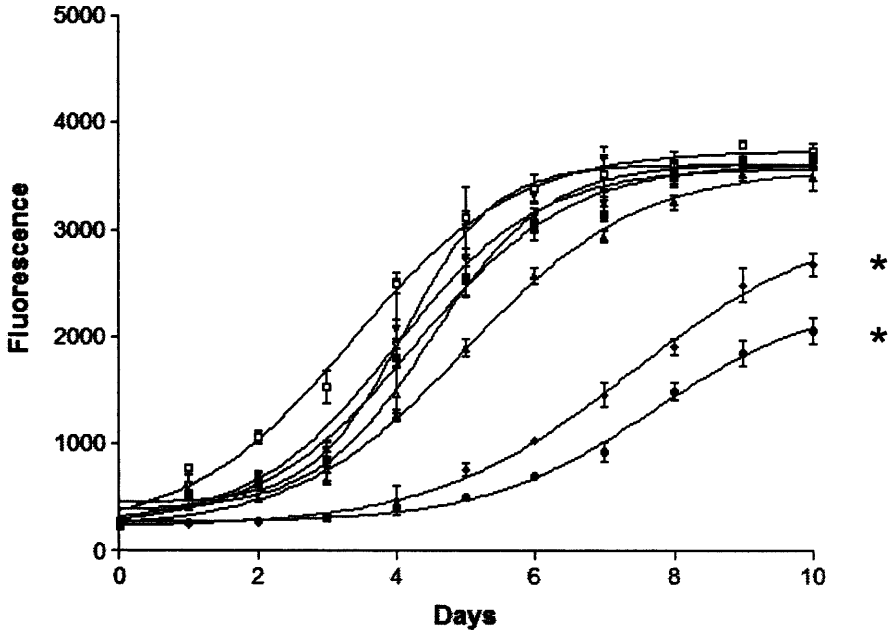


Figure 6.2: *In vitro* proliferation of 8 WT clones
Cells were plated at $1.5x10^4$ per well in triplicate and incubated daily with 10% Alamar Blue solution for two hours. Fluorescence was recorded and plotted over 10 day period. Data are representative of 2 separate experiments. * represents cell line excluded from further experiments.

particularly fast rate. Similar observations were made in $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ clones as shown in Figures 6.3, 6.4 and 6.5 respectively. Several lines were identified as differing from the dominant normal growth pattern within the same group. Those clones with abnormal growth curves were not used for further experiments - these are denoted by * on Figures 6.2-6.5. Data shown is representative of two separate experiments. Subsequently, three clones were identified from each of WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ groups which showed comparable and reproducible growth curves. The 12 selected clones were re-tested for proliferative rates with all shown to grow at comparable rates *in vitro*. These data are shown in Figure 6.6 and confirm suitability of these clones for further experiments.

6.4 Characterisation of clones

6.4.1 CReg characterisation by flow cytometry

As described in Chapter 1, tumour cells are often found to express high levels of one, or several CReg proteins. These proteins are known to be important in protecting cells from C and enhanced tumour killing has been achieved by down-regulation (Zell et al. 2007) or blockade (Di Gaetano et al. 2003) of these proteins. As such, expression of CReg is an important parameter likely to have implications in C susceptibility and *in vivo* growth of these cell lines. Differences between groups in the CReg molecules and levels expressed were important considerations for further experiments and must have implications when analysing the behaviour of these cells in response to C. Data described and discussed in Chapter 5 highlighted a major difference in CReg expression between heterogeneous populations of WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ tumour cells. The expression or lack of Crry and CD55 was consistent between groups, however, only tumours excised from $C3^{-/-}$ animals were found to express CD59. In contrast, expression of CD59, and thus the ability to regulate the terminal pathway of C was not observed in tumours excised from WT, $C1q^{-/-}$ or $CD55^{-/-}.CD59^{-/-}$ mice.

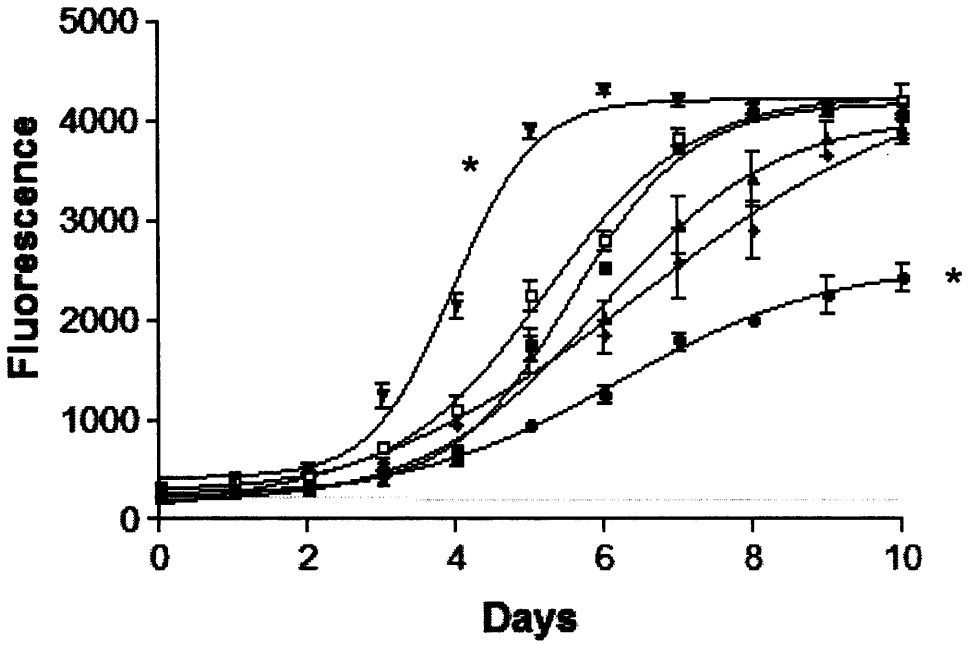


Figure 6.3: ***In vitro*** **proliferation of 8 C1q$^{-/-}$ clones**
Cells were plated at 1.5x10^4 per well in triplicate and incubated daily with 10% Alamar Blue solution for two hours. Fluorescence was recorded and plotted over 10 day period. Data are representative of 2 separate experiments. * represents cell line excluded from further experiments.

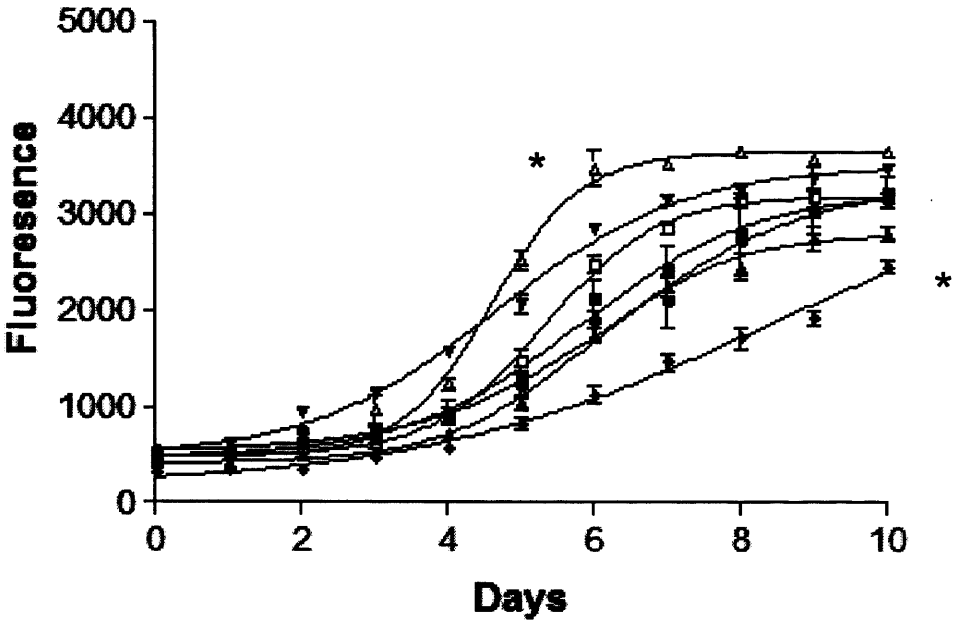


Figure 6.4: ***In vitro*** **proliferation of 8 C3$^{-/-}$ clones**
Cells were plated at 1.5x10^4 per well in triplicate and incubated daily with 10% Alamar Blue solution for two hours. Fluorescence was recorded and plotted over 10 day period. Data are representative of 2 separate experiments. * represents cell lines excluded from further experiments.

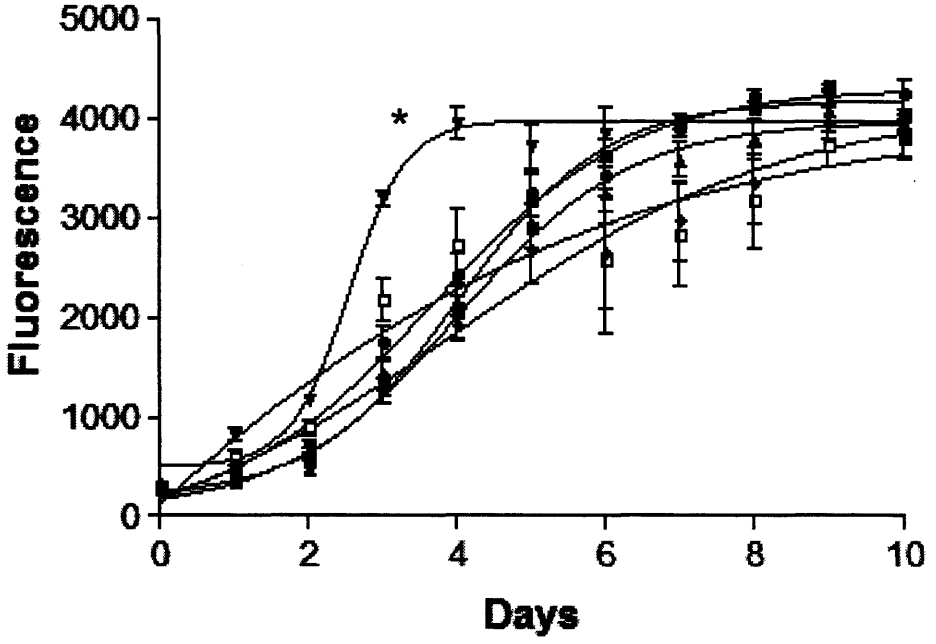


Figure 6.5: ***In vitro*** **proliferation of 8 CD55$^{-/-}$.CD59$^{-/-}$ clones**
Cells were plated at 1.5x10^4 per well in triplicate and incubated daily with 10% Alamar Blue solution for two hours. Fluorescence was recorded and plotted over 10 day period. Data are representative of 2 separate experiments. * represents cell lines excluded from further experiments.

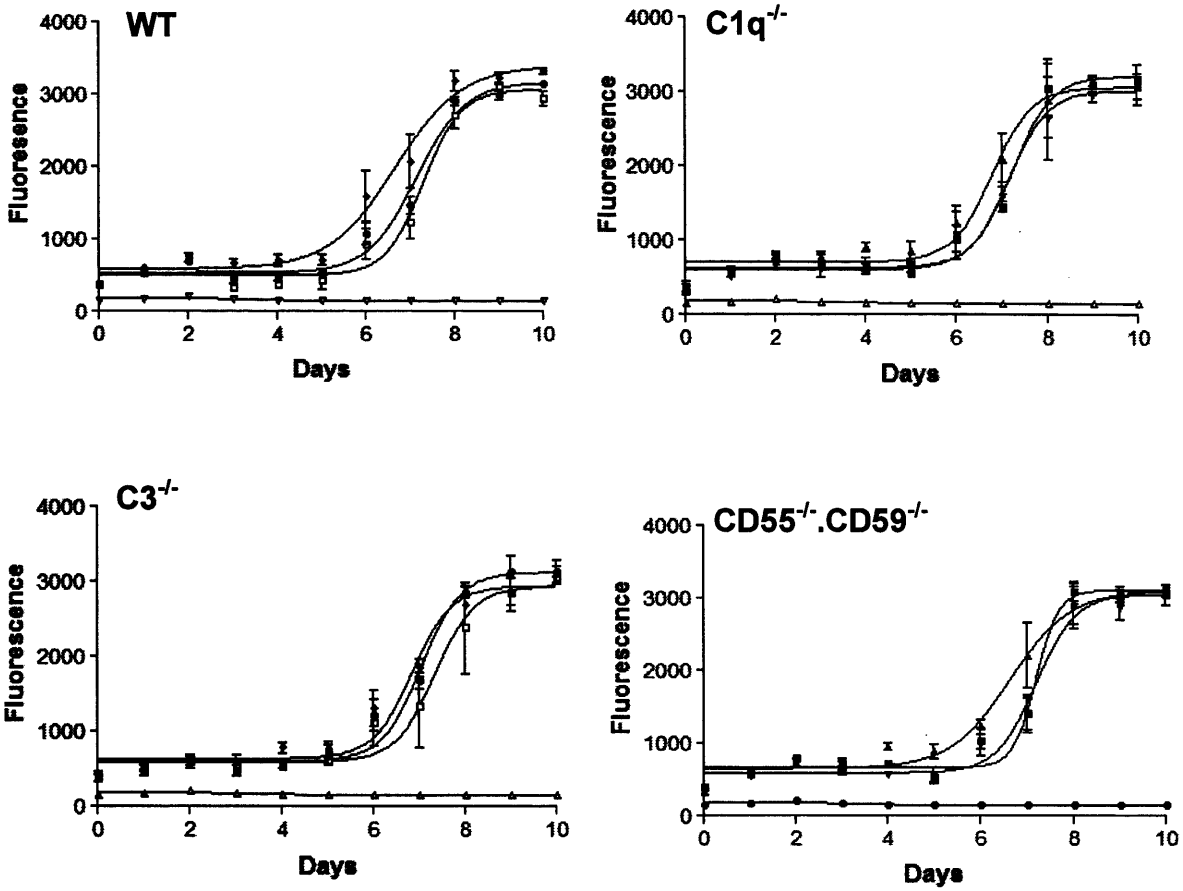


Figure 6.6: ***In vitro*** **proliferation of 3 WT, C1q$^{-/-}$, C3$^{-/-}$ and CD55$^{-/-}$.CD59$^{-/-}$ clones**
3 clones per group were selected and plated at 1.5x10^4 per well in triplicate. Populations were incubated daily with 10% Alamar Blue solution for two hours. Fluorescence was recorded and plotted over 10 day period. Control wells contained no cells.

Novel clonal cell lines were selected and expanded from each tumour genotype as described above. These populations were characterised for CReg expression (CD55, CD59, Crry) via flow cytometry as fully described in Chapter 2. Cells were stained with appropriate antibodies against CD55, CD59 and Crry with fluorescence noted and compared to control populations. As with previous experiments, mouse erythrocytes were included as a positive control. These cells are known to express high levels of each of these CReg (Miwa et al. 2002) and were useful to confirm the functionality of the antibodies used. These data are summarised in Figure 6.7 and confirmed the presence of CD55, CD59 and Crry on erythrocytes. This is evidenced by a clear shift in median fluorescence values following staining with MD1 (anti-CD55), Mel-4 (anti-CD59) and 5D5 (anti-Crry) monoclonal antibodies. Each was detected by staining with RPE-conjugated donkey anti-rat IgG secondary Ab and MFI values noted and compared to control (unstained) populations.

Figure 6.8 shows summarised flow cytometry data from the 12 clones. In each case, the shift in median fluorescence intensity (MFI) between stained and control cells was plotted allowing quantification of protein expression for each line. Figure 6.9 displays the mean and standard deviation of shifts observed for each genotype and each CReg. CD55 was not detected on any of the cell lines as was repeatedly found and described in Chapters 4 and 5. Crry was detected at high levels on all cells analysed with seemingly equivalent levels observed for each genotype. This was evidenced by a consistent mean shift (64-72 MFI units) for each genotype and no discernable difference between data in different groups. However, a clear difference was found in terms of CD59 expression with none detected in WT, $C1q^{-/-}$ or, as expected, in $CD55^{-/-}$.$CD59^{-/-}$ cell lines. Conversely, each of the three clonal lines deficient in C3 expressed significant amounts of CD59. The observed change in median was seen only in $C3^{-/-}$ clones and there was found to be a statistically significant difference between this group and each of the others ($p=0.005$).

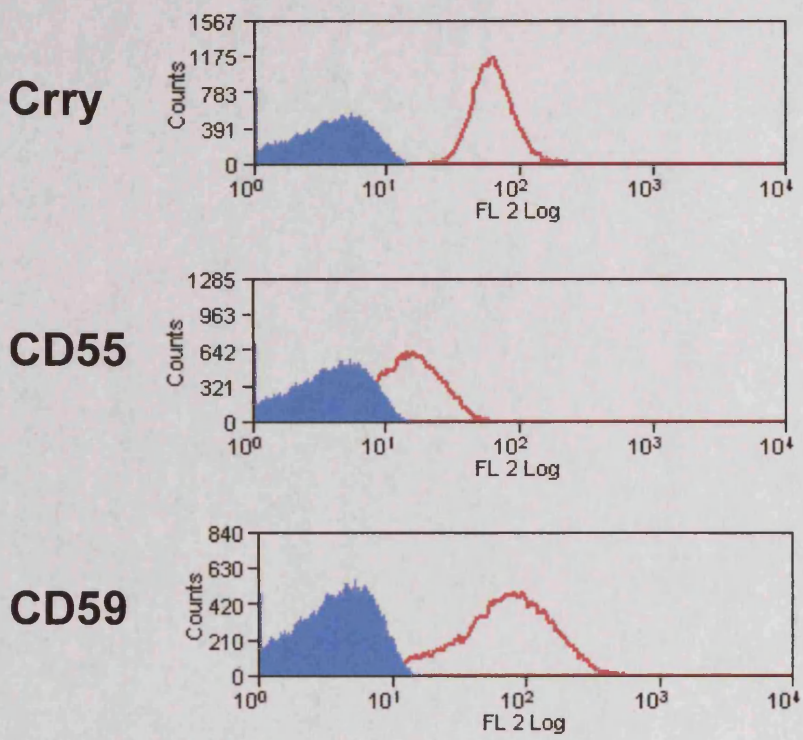


Figure 6.7: CReg expression on mouse erythrocytes
Mouse erythrocytes were stained using CD55, CD59 and Crry specific Abs. A secondary donkey anti-rat-RPE Ab was used to visualise bound Ab and is plotted in red. Control cells stained with secondary Ab only are also shown as the blue population.

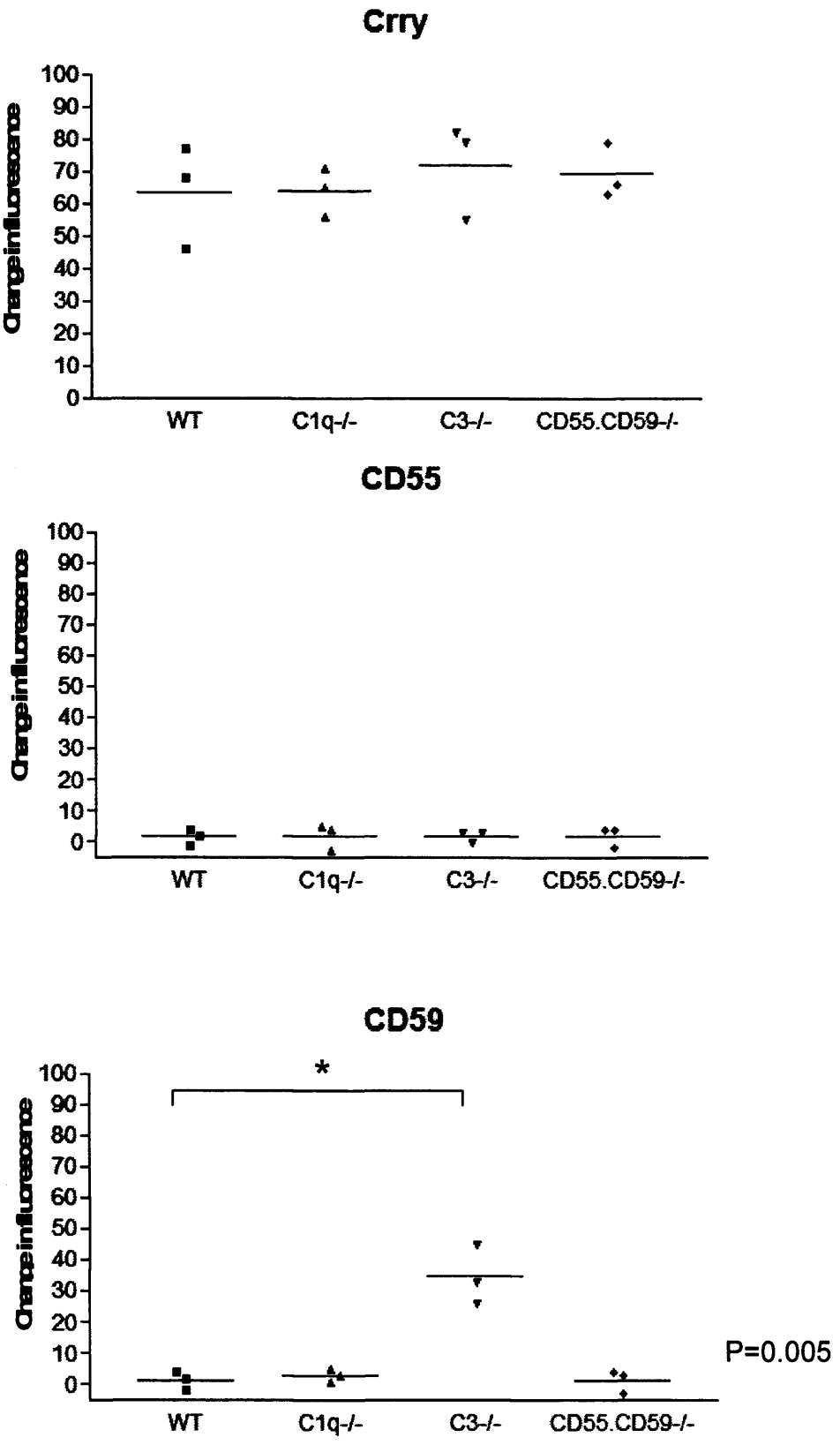


Figure 6.8: CReg expression on 3 WT, C1q$^{-/-}$, C3$^{-/-}$ and CD55$^{-/-}$.CD59$^{-/-}$ clones
Clonal lines were stained using CD55, CD59 and Crry specific Abs. A secondary donkey anti-rat-RPE Ab was used to visualise bound Ab. Control cells stained with secondary Ab only were used to calculate shift in median fluorescence. Data were analysed using the unpaired students t-test. * represents $p<0.05$

	Crry	CD55	CD59
WT	64 ± 16	1 ± 3	1 ± 3
C1q$^{-/-}$	64 ± 8	2 ± 4	3 ± 2
C3$^{-/-}$	72 ± 15	2 ± 2	35 ± 10
CD55$^{-/-}$.CD59$^{-/-}$	69 ± 9	2 ± 3	1 ± 4

Figure 6.9: Mean CReg expression on 3 WT, C1q$^{-/-}$, C3$^{-/-}$ and CD55$^{-/-}$.CD59$^{-/-}$ clones
Shifts in median fluorescence intensities were recorded for 3 clones per group. Data shown include mean ± SEM.

6.4.2 ELISA characterisation of C3/C3b/iC3b present in s/n

A hypothesis integral to this thesis is that tumour cells may utilise C to aid their proliferation or metastasis. Cancer-derived cell lines have previously reported to synthesise and secrete their own C3 into the tumour micro-environment (Jean et al. 1997, Kitano and Kitamura 1993) while fibroblasts are also known to produce the C3 protein (Katz, Revel and Strunk 1989). Therefore a potentially important characteristic to assess is the secretion of C3 by these fibrosarcoma lines. An ELISA method was used to detect C3 released into tissue culture s/n, additionally presence of the activation product of C3 cleavage (C3b) and the inactivated form of this protein (iC3b) was also tested using the mAb described in previous Chapters. Cells were cultured to ~40% confluence, fresh media added and incubated for 48 hours. s/n was removed from each population (approx $5x10^6$ cells), spun to remove cellular debris and concentrated 10-fold via centrifugation through a 0.22µm centrifugal concentrator. s/ns were assayed in triplicate for C3/C3b/iC3b presence using a plate-bound rat monoclonal Ab specific for these proteins. Bound protein was detected using a rabbit polyclonal anti mouse C3 Ab and a peroxidase conjugated donkey anti-rabbit IgG Ab. A range of known C3 concentrations was assayed on the same plate comprising a titration of purified mouse C3 concentrations. These known concentrations were used to create a standard curve against which s/n readings could be compared, whilst fresh cell culture medium was included as a negative control. Additionally, purified mouse C3 (100ng/ml) was added to culture media as a positive control to ensure that positive signal could be detected and was not inhibited by culture media. A positive signal was recorded from the C3 'spiked' sample, and corresponded to a concentration of ~92ng/ml when compared to the standard curve.

Figure 6.10 shows a standard curve of C3 concentrations and allowed accurate detection of C3 concentrations in the range 15-1200ng/ml. However, absorbance readings obtained following analysis of tissue culture s/n from 3 clonal cell populations (from WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$) were below the threshold of detection in this assay. Figure 6.11 shows levels of C3 as

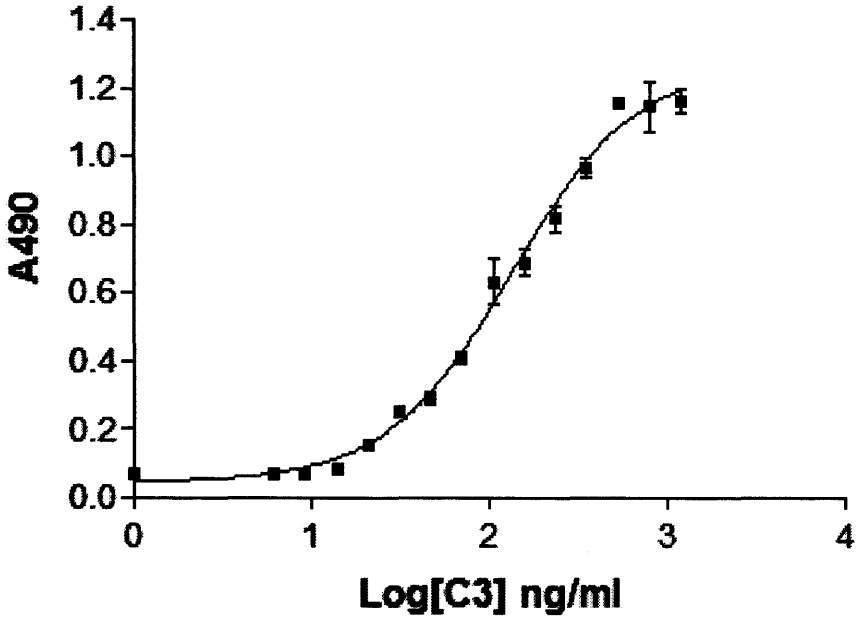


Figure 6.10: C3/C3b/iC3b detection by ELISA
96 well plates were coated with a monoclonal rat anti-mouse C3 Ab and incubated with a serial dilution of known concentrations of purified mouse C3. Plates were washed before incubation with polyclonal rabbit anti-mouse C3 Ab. Bound antibody was detected using HRPO-conjugated anti-rabbit IgG and visualised using an OPD preparation. Dilutions of C3 were assayed in triplicate.

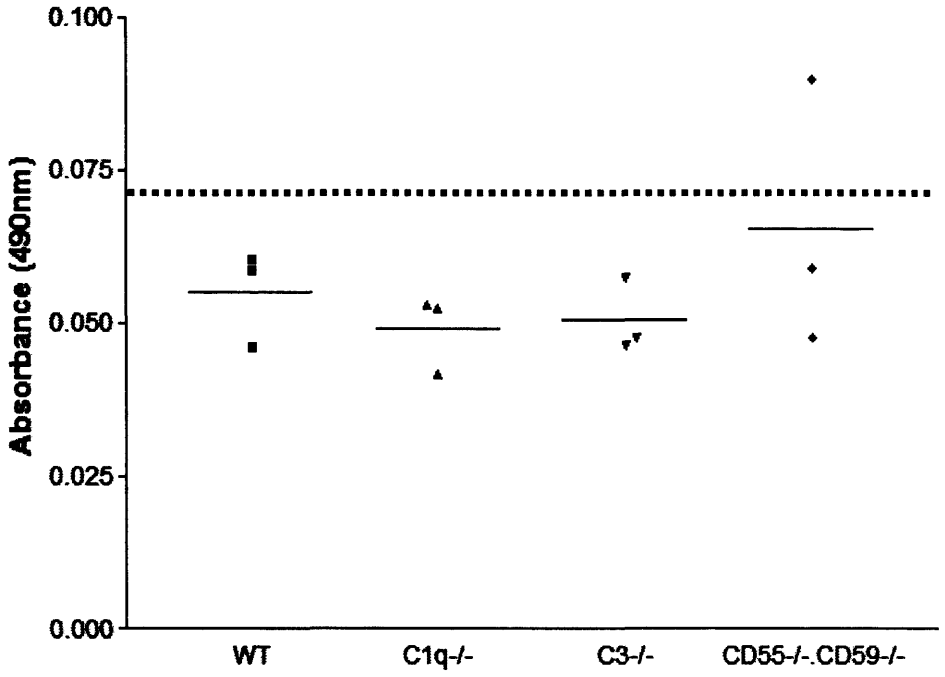


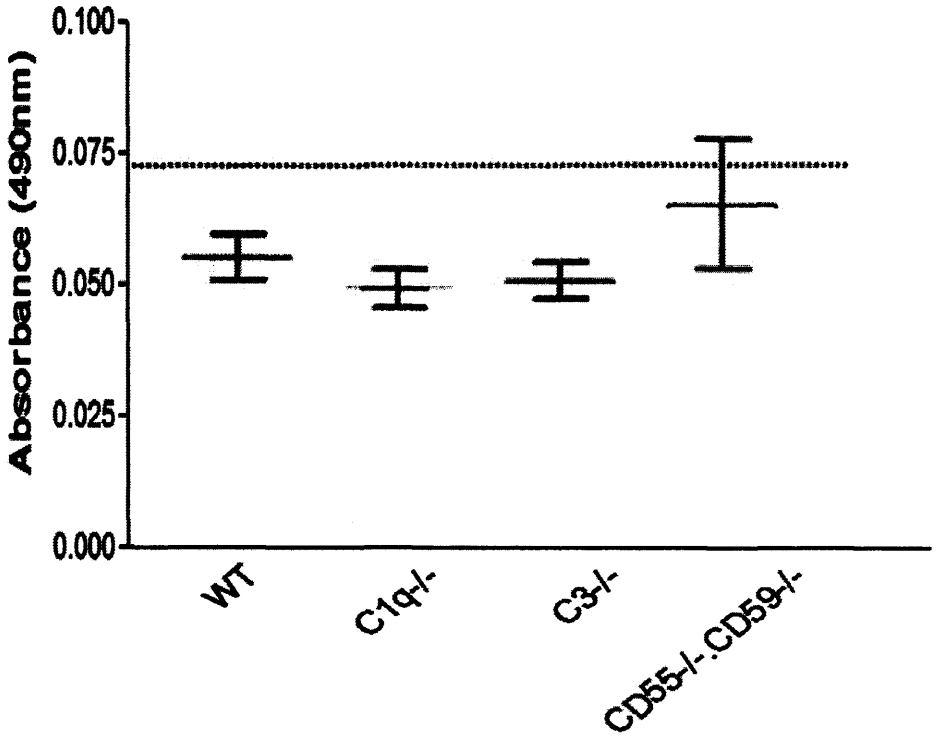
Figure 6.11: Assessment of C3/C3b/iC3b by ELISA in cell culture supernatants
96 well plates were coated with a monoclonal rat anti-mouse C3 Ab and incubated with tissue culture supernatant from 3 clonal lines from each genotype. Supernatant was concentrated 10-fold prior to assay. Plates were washed before incubation with polyclonal rabbit anti-mouse C3 Ab. Bound antibody was detected using HRPO-conjugated anti-rabbit IgG and visualised using an OPD preparation. Supernatant from each clone was assayed in duplicate. Horizontal bars represent means of the 3 clones per genotype while horizontal dashed bar represents minimal detectable level of C3 15ng/ml. Data are representative of 2 separate experiments.

assessed by ELISA with the dashed line representing the C3 detection threshold (15ng/ml). All mean values corresponding to clonal cell lines were below this detection threshold. One clone of $CD55^{-/-}.CD59^{-/-}$ genotype exhibited a reading marginally above the threshold but exhibited high variation between triplicate values and was not reproducible. Figure 6.12 indicated mean ± SEM values for each genotype. A previous study reporting C3 secretion *in vitro* by a human melanoma population found 350ng $C3/10^6$ cells/24 hours. This value is significantly higher than the observed values for fibrosarcoma lines measured in this assay despite increased cell numbers, incubation time and s/n concentration. These data suggest no C3/C3b or iC3b is present in tissue culture media incubated with clonal fibrosarcoma lines. A mean control value of 0.058 was recorded for fresh media alone indicating the Ab used did not cross-react with bovine C3 in tissue culture media.

Heterogeneous (parent) cell populations were also assayed for C3 secretion as described above. Use of heterogeneous (multi-cellular origin) populations increases the probability of the population containing C3-secreting cells. However, levels of C3/C3b/iC3b detected in these populations depends on the proportion of C3-producing cells in addition to amounts secreted per cell. Figure 6.13 shows a standard curve of known C3 concentrations with a minimum C3 concentration detection level of 20ng/ml. The absorbance values also shown in Figure 6.13 correspond to C3 concentrations below this level and substantiate the previous finding further suggesting that negligible or no C3, C3b or iC3b is present in tissue culture s/n incubated with the tumour cells over a 48 hour period.

6.4.3 RT-PCR characterisation of C3, CD55 and CD59 mRNA levels

Convincing data exist to support the over-expression of protective proteins on tumour cells *in vivo* e.g. CD55 (Varsano et al. 1998) and CD59 (Kuraya et al. 1992). CD55 was not detected on fibrosarcoma surfaces either in clonal or parent lines. CD59 was detected only on $C3^{-/-}$ lines, while ELISAs described above did not detect secreted C3 (or associated cleavage products) by clonal or parental lines. However, analysis of mRNA can provide a quantitative and



	Mean ± SEM
WT	0.055 ± 0.010
$C1q^{-/-}$	0.049 ± 0.012
$C3^{-/-}$	0.051 ± 0.007
$CD55^{-/-}.CD59^{-/-}$	0.066 ± 0.025

Figure 6.12: Assessment of C3/C3b/iC3b by ELISA in cell culture supernatants
Mean absorbance levels were calculated from 3 clones per group following ELISA. Data shown represents mean ± SEM. Dashed line represents threshold for detection.

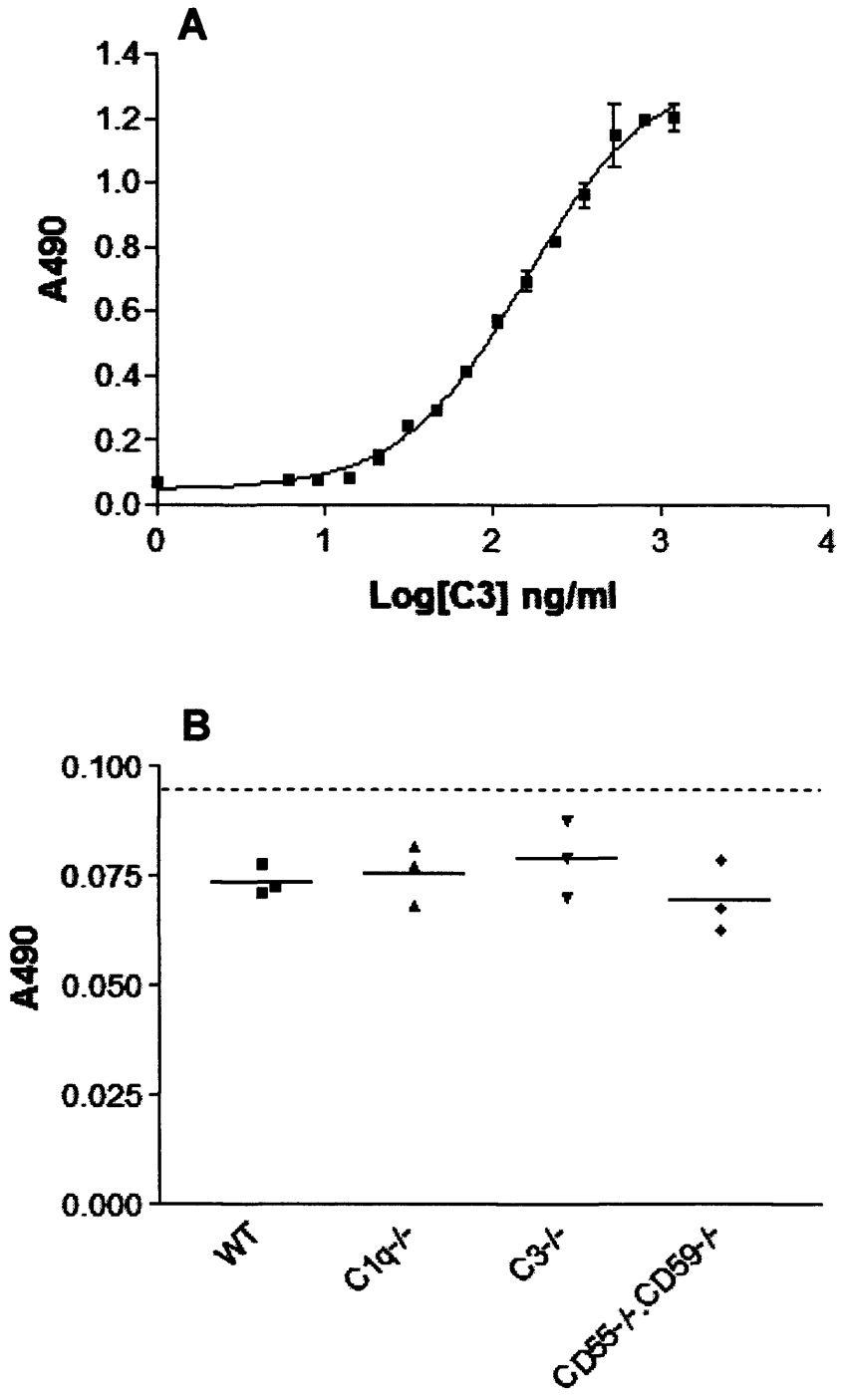


Figure 6.13: Assessment of C3/C3b/iC3b by ELISA in parent cell culture supernatants

96 well plates were coated with a monoclonal rat anti-mouse C3 Ab and incubated with purified C3 concentrations (panel A) or tissue culture supernatant from 3 heterogeneous lines from each genotype (panel B). Supernatant was concentrated 10-fold prior to assay. Plates were washed before incubation with polyclonal rabbit anti-mouse C3 Ab. Bound antibody was detected using HRPO-conjugated anti-rabbit IgG and visualised using an OPD preparation. Supernatant from each cell population was assayed in duplicate. Horizontal bars represent means of the 3 clones per genotype while horizontal dashed bar represents minimal detectable level of C3 20ng/ml.

highly sensitive measure of gene transcription regardless of protein expression. Detection of CReg or C3 mRNA could point to intra-cellular stores of these proteins which would be undetectable by the above methods. Assessment of mRNA levels for these proteins was undertaken via quantitative real-time PCR (RT-PCR) as fully described in Chapter 2. Briefly, mRNA was isolated from cell cultures and converted to cDNA via reverse transcription. Primers specific for CD55, CD59 and C3 were used to identify and amplify specific sequences. Included in the PCR procedure is a fluorescent dye (SYBR® Green) which is measured in real time following each amplification cycle allowing quantification of DNA concentrations. A preparation of fresh mouse liver was included as a positive control. Hepatocytes are known to synthesise C3 and express CD55 and CD59 (Funabashi et al. 1994, Song et al. 1996). Each population tested was also assayed for amplification of a housekeeping gene (β-actin), known to be uniformly expressed in cells and used to normalise detected levels between samples. For each population and gene, a Ct (cycle threshold) value is produced and represents the number of cycles required for fluorescence to reach a pre-determined threshold. Included in the experiment are negative controls for C3 mRNA ($C3^{-/-}$ lines) and CD55 and CD59 ($CD55^{-/-}.CD59^{-/-}$ lines). These lines were used as a baseline and Ct values referenced against this value and used to calculate relative gene expression in each population.

Data shown in Figure 6.14 closely correspond with the flow cytometry and ELISA data described previously. Graph A shows no significant CD55 mRNA present in any clones, graph B shows CD59 mRNA present in $C3^{-/-}$ lines ($p=0.008$) only while no C3 mRNA was detected in any clones assayed (C). C3, CD55 and CD59 mRNA was detected at high levels in hepatocyte cells. Almost identical findings were observed following analysis of data from parent populations (n=3). These data are shown in Figure 6.15 and mirror the previous findings. Again, no CD55 or C3 mRNA was detected in any fibrosarcoma populations analysed.

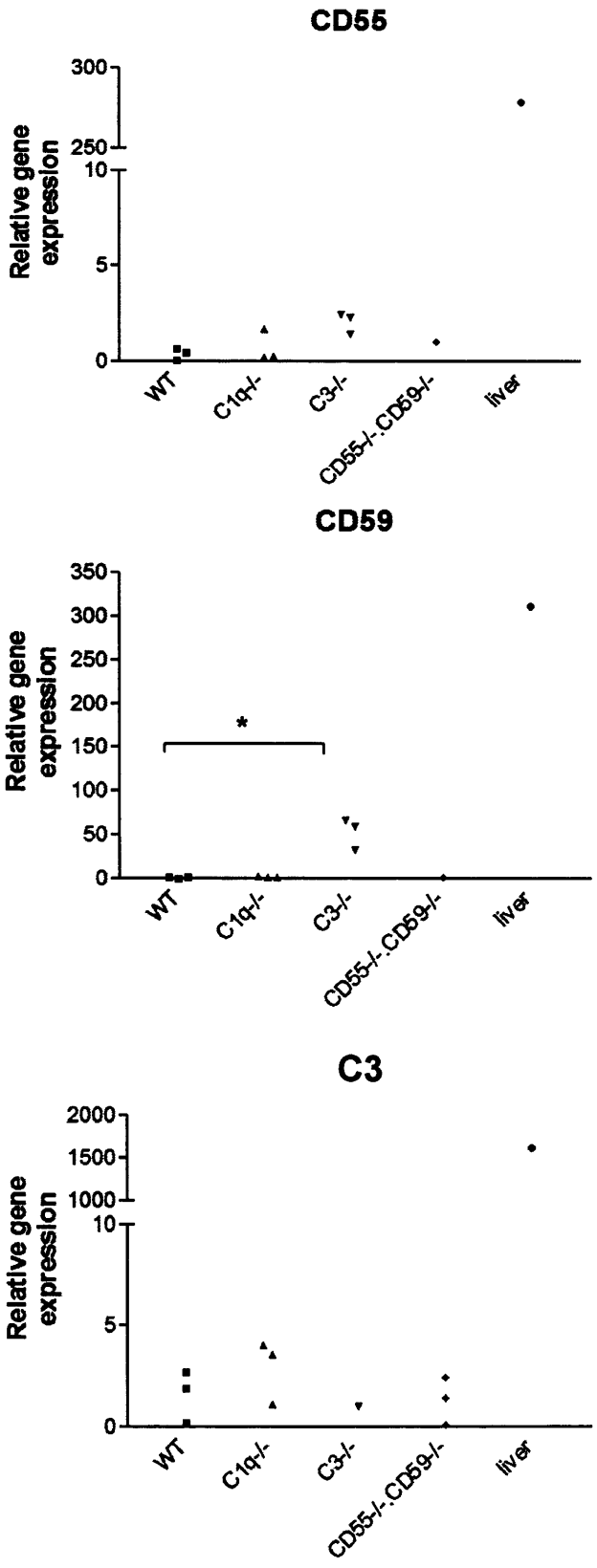


Figure 6.14: RT-PCR analysis of CD55, CD59 and C3 mRNA in clonal populations
mRNA was extracted from clonal cell populations or fresh liver sample. RNA was converted to cDNA via reverse transcription. Primer sequences specific for mouse CD55, CD59, C3 and β-actin were used to amplify sequences in presence of Sybr-Green fluorescent dye. Fluorescence levels per cycle were recorded and used to calculate C(t) values for each protein per cell line. Reference gene data (β-actin) was deducted and values expressed relevant to a population deficient in relevant gene. Data were analysed using a non-paired students t-test. * indicates a p-value <0.05

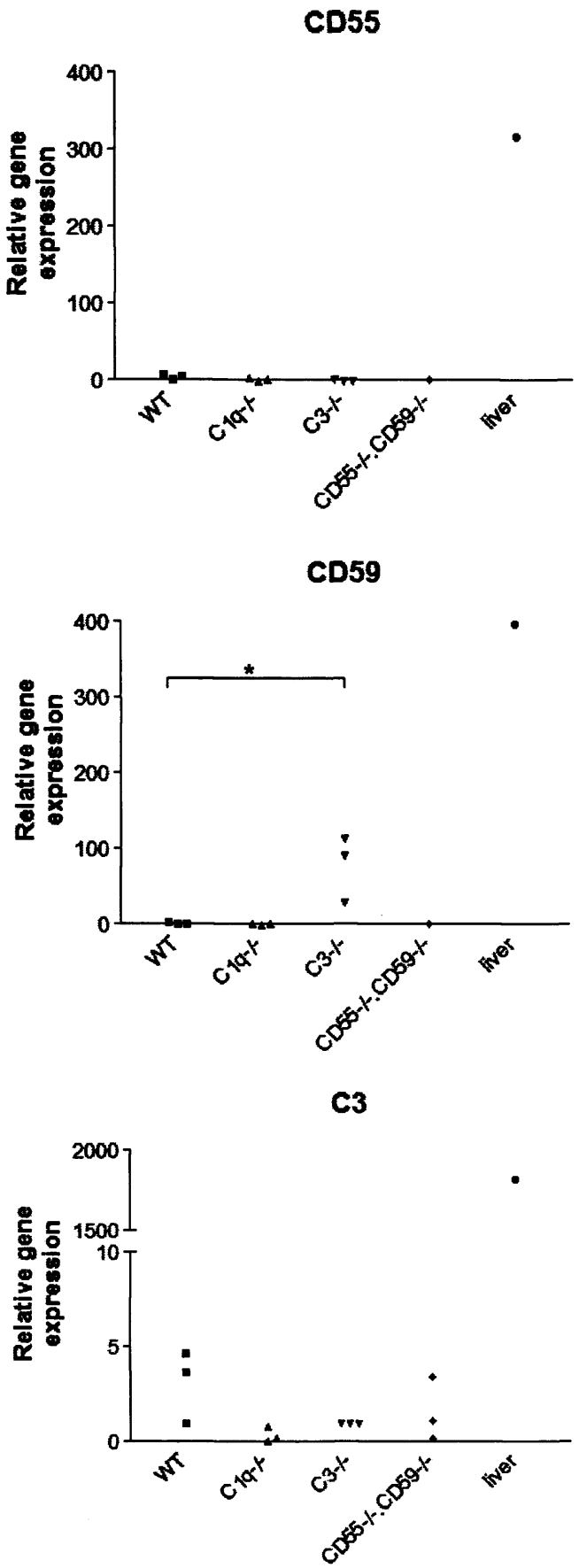


Figure 6.15: RT-PCR analysis of CD55, CD59 and C3 mRNA in heterogeneous populations mRNA was extracted from heterogeneous cell populations or fresh liver sample. RNA was converted to cDNA via reverse transcription. Primer sequences specific for mouse CD55, CD59, C3 and β-actin were used to amplify sequences in presence of Sybr-Green fluorescent dye. Fluorescence levels per cycle were recorded and used to calculate C(t) values for each protein per cell line. Reference gene data (β-actin) was deducted and values expressed relevant to a population deficient in relevant gene. Data were analysed using a non-paired students t-test. * indicates $p<0.05$.

6.5 *in vitro* sensitivity to complement

In order to assess the relative sensitivities of clones to C, a 'killing' assay was used based on classical pathway activation. Variation between populations in susceptibility to C-mediated lysis may highlight important differences between groups and provide useful indicators by which *in vivo* growth can be predicted or assessed. Cells were sensitised, incubated with various titres of C and treated with 4µg/ml propidium iodide (P.I.) solution. P.I. binds tightly to DNA strands and emits a strong fluorescent signal, detectable by flow cytometry. Only dead/dying cells with compromised cell membranes allow the passage of P.I. into the cell and alive/healthy cells do not exhibit fluorescence. The sensitising Ab used was a rabbit anti-mouse erythrocyte Ab and as depicted in Figure 6.16 binds to these tumour cell lines at high levels as detected by RPE-conjugated donkey anti-rabbit IgG Ab. However, despite using extremely high titres of a polyclonal sensitising Ab and neat mouse serum, negligible killing of clones could be detected. As shown in Figure 6.17, the maximal lysed population makes up just 2% of the entire cell count suggesting that these cells are resistant to C-mediated cytotoxicity or that the assay is ineffective. In order to verify the lytic capability of serum used in the assay, serum was also assayed for activity against sensitised mouse erythrocytes as shown in Figure 6.18. Erythrocytes were readily lysed by low doses of C as evidenced by haemoglobin release. This indicates that the serum is functionally active and that the Ab chosen is capable of activating the classical pathway.

Cell populations were observed to aggregate during the repeated wash/re-suspend steps involved with the assay described above. Cells were observed to aggregate and required vigorous and lengthy shaking to re-obtain a single cell suspension. Fibrosarcoma cells are naturally adherent, while this observation may also be attributed to an effect known as agglutination whereby cells 'clump' together following Ab fixation. Consequently, an alternative assay was developed to determine C-susceptibility in which cells remained adherent to a surface throughout the protocol. Cells were grown to 50-60% confluency on 6-well plates and loaded with calcein acetoxymethyl

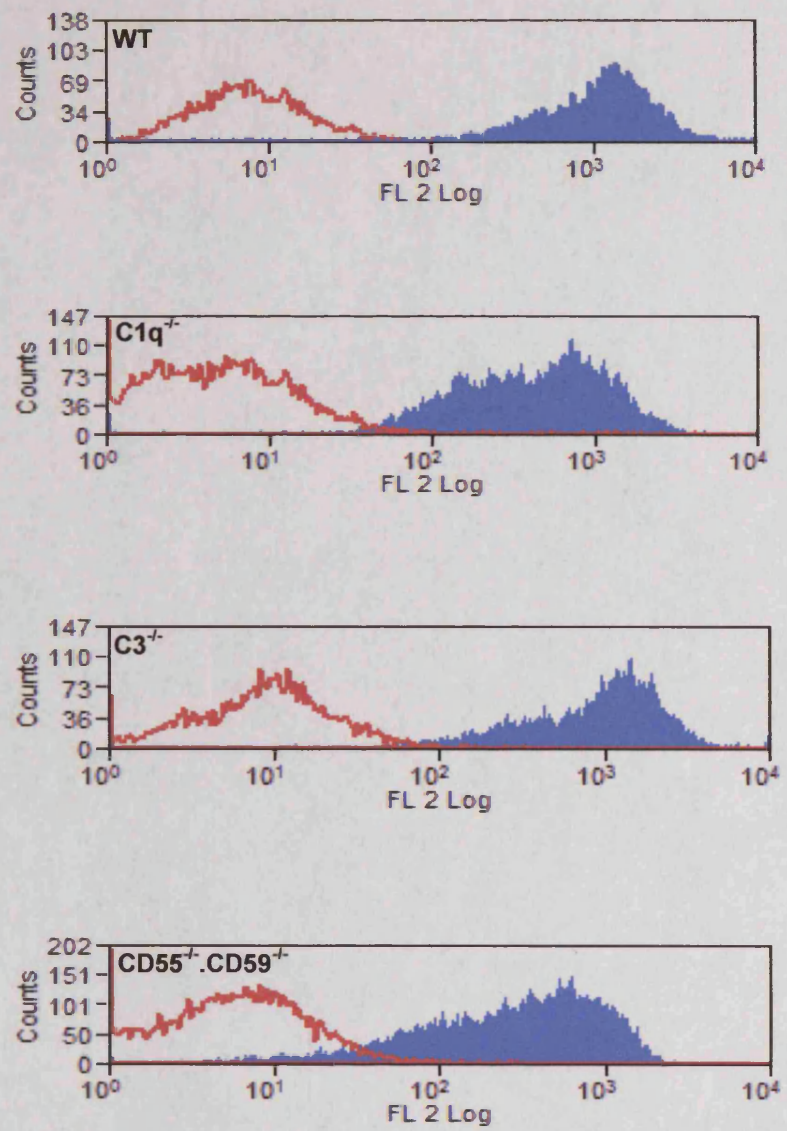


Figure 6.16: Binding of sensitising Ab to fibrosarcoma populations
Clonal populations of known genotypes were incubated with polyclonal rabbit anti-mouse erythrocyte Ab. Bound Ab was detected using RPE-conjugated donkey anti-rabbit IgG. Red lines represent clonal populations incubated with secondary Ab only. One representative clone is shown from each group comprising three separate clonal lines.

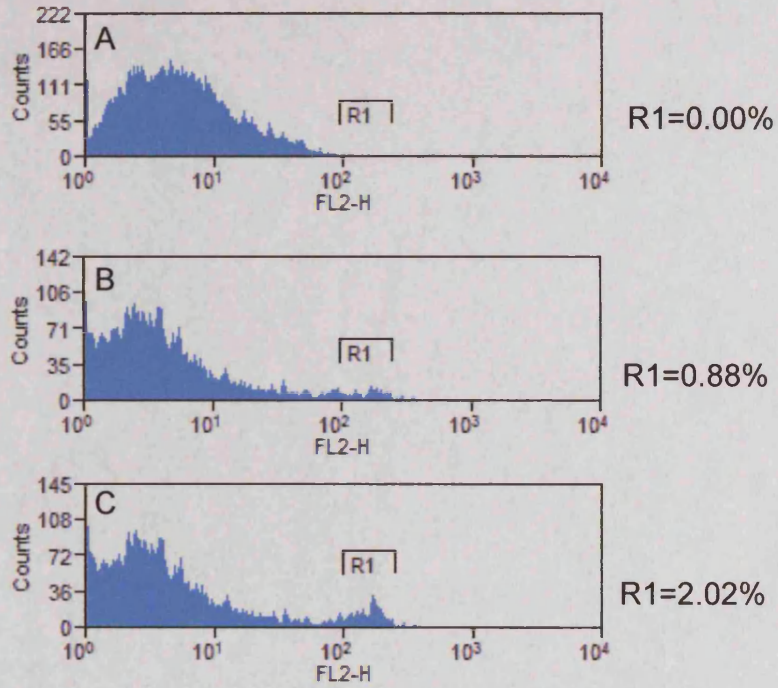


Figure 6.17: Propidium Iodide staining of cells following CP activation
Cells were sensitised with 6.67% polyclonal rabbit anti-mouse erythrocyte antibody, before incubation with normal mouse serum at varying dilutions. Lysed cells were stained using propidium iodide and analysed using flow cytometry. Panel A, B and C depict cells treated with 10%, 50% and 100% rat serum. Region 1 (R1) contains cells staining positive for propidium iodine and represents 'dead' population.

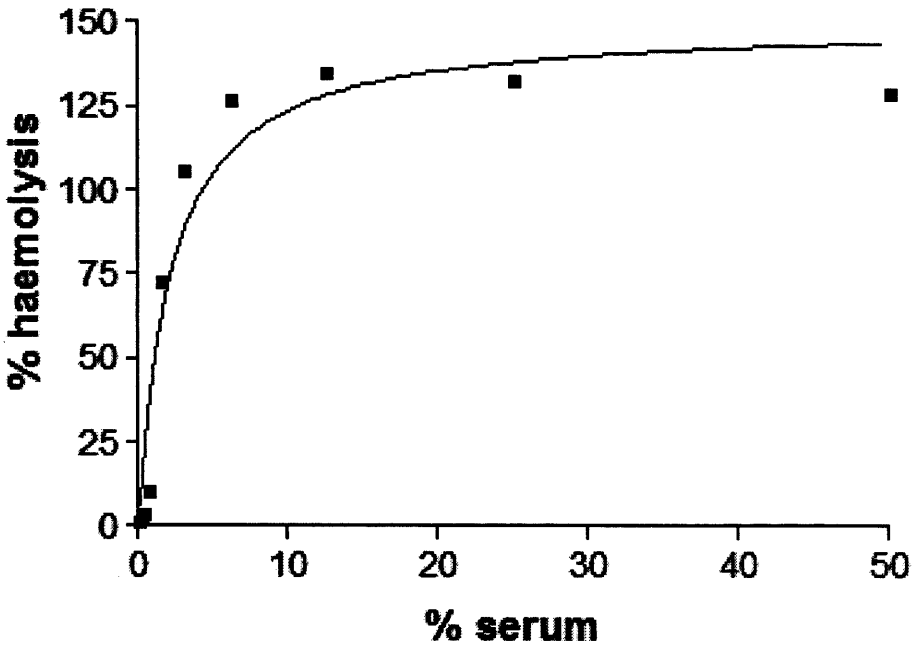


Figure 6.18: Haemolysis of mouse erythrocytes following CP activation
Erythrocyte cells were sensitised using 2% polyclonal rabbit anti-mouse Ab. Cells were washed and incubated with a range of mouse serum dilutions and haemolysis calculated by reference to 0% and 100% lysis values.

ester (Calcein-AM). This compound is readily transported across plasma membranes where it is cleaved by cytosolic esterases to free calcein – a fluorescent dye which is not membrane permeable. Cells were sensitised, washed and attacked *in situ* with titres of fresh mouse sera. Dye release was measured by recording fluorescence using 490nm excitation and 530nm emission filters, thus providing a quantitative assessment of cell death.

Figure 6.19 shows representative data from 1 clone from each genotype: WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$. The graph depicts fluorescence following C-mediated lysis at a range of sera dilutions and provides a measure of calcein release proportional to the number of dead cells. Additionally, intact cells remaining impermeable and retaining calcein were treated with 0.5% detergent. This step released remaining calcein, fluorescence was again recorded to provide a measure of cells surviving C-attack. Fluorescent signal released by C-mediated lysis (bottom line) and signal released by detergent (top line) are plotted. Wells in which populations were previously exposed to high C titres exhibited lower fluorescence following detergent-mediated lysis. Combining these data allowed calculation of % lysis for each cell population whereby calcein released by C can be determined as a proportion of the total calcein release (C-mediated and detergent-mediated lysis combined). Data collected from clonal cell populations are presented in Figure 6.20. Graph A depicts % lysis data following treatment of populations with 0-100% mouse serum. Each curve represents one genotype with each following a similar pattern. The curves tended towards a maximal killing value (K_{max}) value of 25-30% lysis with WT and $C3^{-/-}$ lines appearing to lyse less readily than $C1q^{-/-}$ or $CD55^{-/-}$. $CD59^{-/-}$. However, the observed differences were slight while each population exhibited a high background lysis with ~18% lysis observed in each population following incubation with 1% BSA/ CFD i.e. in the absence of C. Figure 6.20B shows the same data following subtraction of background lysis from each fluorescence reading. Each curve corresponding to the populations tended towards a plateau i.e. threshold of lysis at a comparable value although the $C3^{-/-}$ curve was peaked slightly lower and the WT curve reached a slightly higher value than that corresponding to $C3^{-/-}$ populations, higher standard errors were also observed in this group. Data shown in this

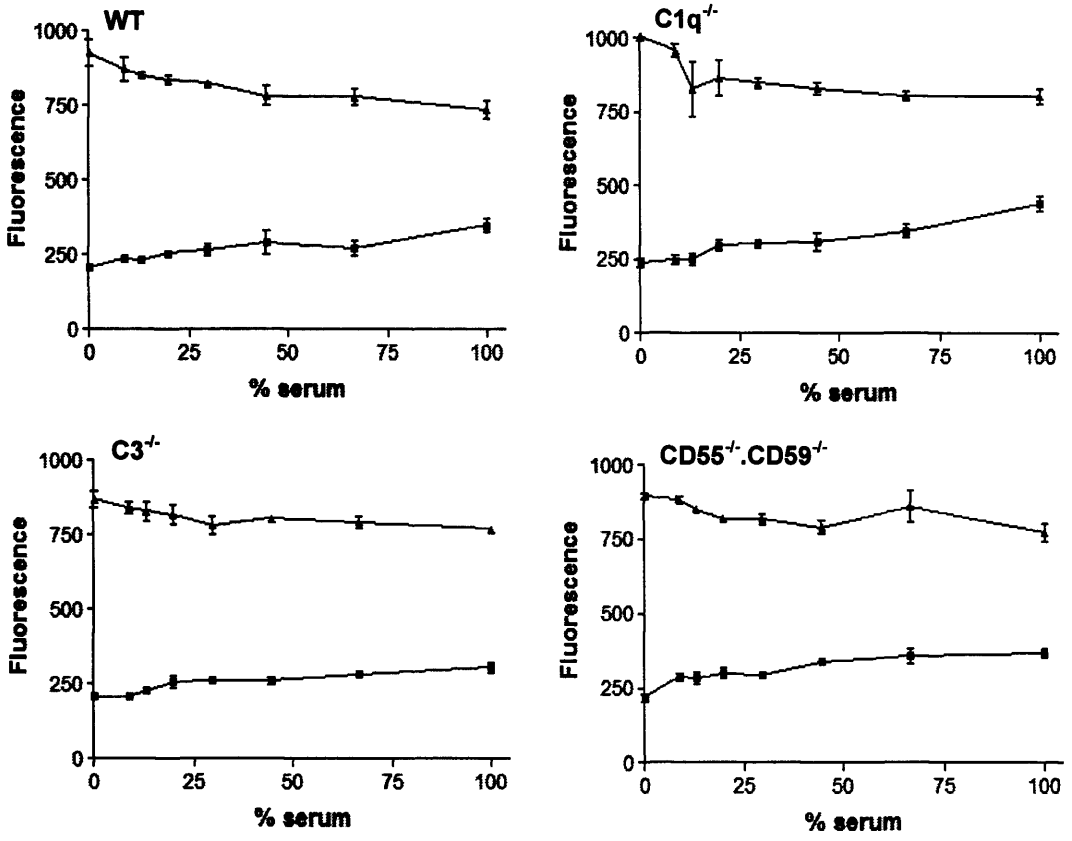


Figure 6.19: Lysis of fibrosarcoma populations via calcein release assay
Cells were pre-loaded with calcein-AM and sensitised with 6.67% polyclonal rabbit anti-mouse erythrocyte Ab. Cells were washed and incubated with a range of mouse serum dilutions. Fluorescence of media was measured. Remaining cells were lysed by detergent and fluorescence re-measured. Complement mediated calcein release is represented by squares while detergent mediated release is represented by triangles. Fluorescence measured at 530nm.

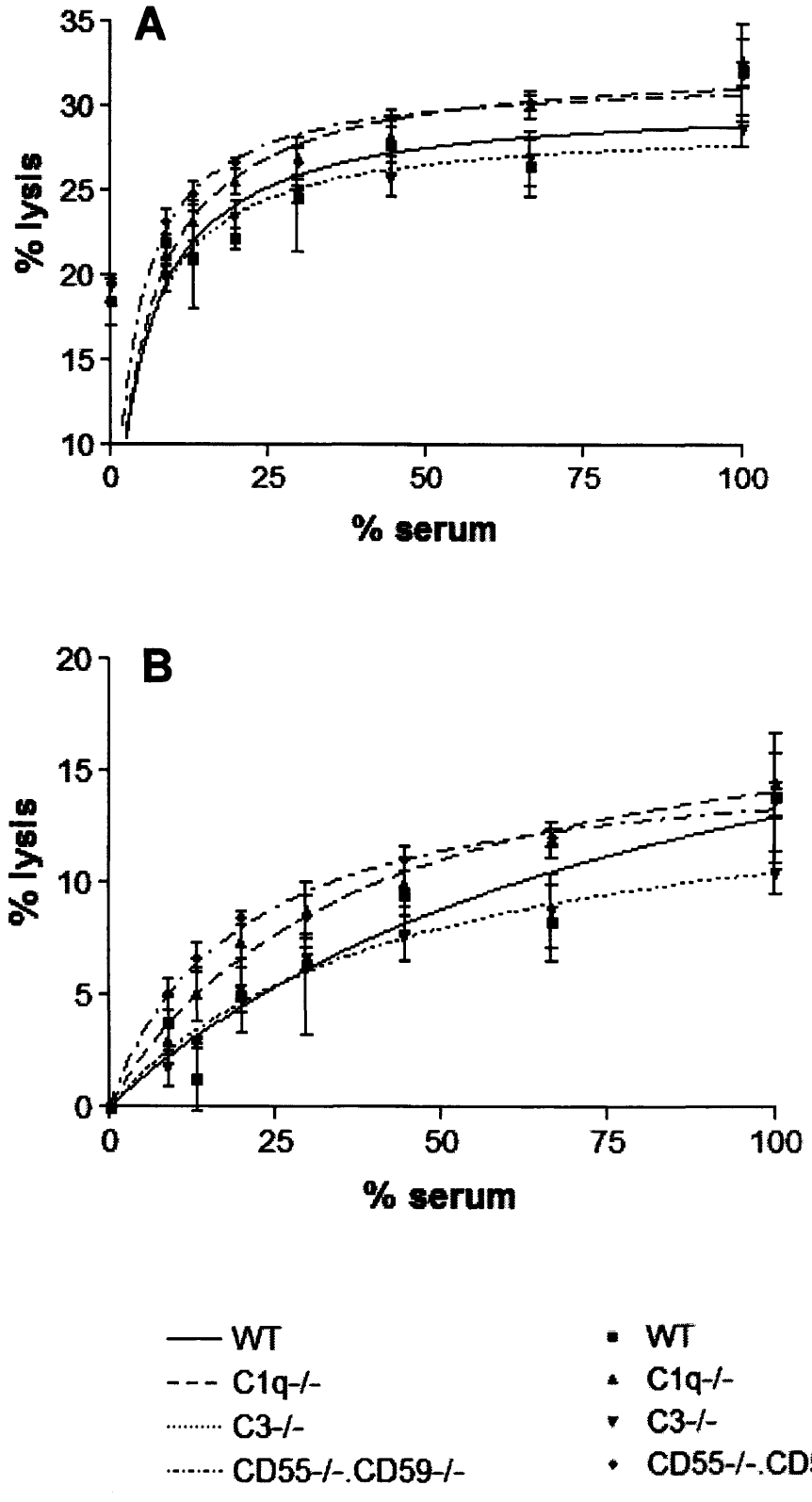


Figure 6.20: Complement mediated lysis of fibrosarcoma populations
% lysis values were calculated as a proportion of total calcein release. Data shown are mean values derived from duplicate assay of three clones per group. Data shown are representative of 2 separate experiments. Panel A represents raw lysis data, panel B represents data following subtraction of background lysis values.

Figure suggest that, while lysis is increased according to serum dilution, C remains ineffective at lysing cells in this system as evidenced by the low maximal percentage lysis values shown in Table 6.2.

	WT	$C1q^{-/-}$	$C3^{-/-}$	$CD55^{-/-}.CD59^{-/-}$
K_{max}	22 ± 9	19 ± 2	14 ± 2	17 ± 1

Table 6.2: Calcein release assay

Maximal killing observed in 3 clonal populations per group. K_{max} refers to maximal killing possible. Mean ± SEM are shown.

The term Kmax refers to the threshold of killing i.e. the plateau reached by each trace, determined using GraphPad Prism software. These observations support data shown in Figure 6.17 suggesting that transformed fibrosarcoma cells are resistant to C mediated lysis.

6.6 C3b/iC3b deposition assay

Data described in the previous section outline difficulties in achieving lysis through C attack on fibrosarcoma lines generated through 3-MCA tumour induction. However, C activation and opsonisation is crucial to many immune and cellular responses as described in Section 1.2. As such, measurement of C3 activation products on cell surface can provide an additional parameter to assess the relationship between C and tumour cells of differing genotypes. A flow cytometric assay was optimised employing the sensitisation and C-activation steps as described above. Following incubation with a range of serum titres, cells were stained for the presence of activated C3 fragments deposited on surfaces by incubation with rat anti-mouse C3 mAb. Fixed anti-C3 mAb was visualised using a donkey anti-rat secondary Ab conjugated to an Alexa-Fluor-594 fluorescent probe. Conventional thinking is that cells better protected against C, or with proficient mechanisms for cleaving or shedding C3 would be expected to have lower levels of C3 (and associated fragments) deposited on its surface. This could predict better survival *in vivo*.

However, an alternative hypothesis is emerging whereby C3 activation and deposition may benefit cells through cellular activation mechanisms described in Section 1.12. In this assay, cells were treated with a maximum dose of 50% serum. This, as shown in previous section, will cause negligible cell death. Accurate analysis of data in this assay requires avoidance of cell death in the experimental populations as these events exhibit irregular forward and side scatter patterns and are not collected by the flow cytometer. It is also possible that dying cells may activate the classical (Taylor et al. 2000), MBL (Nauta et al. 2003) or alternative pathway (Xu et al. 2008). This activation by dead/dying cells could deposit further C3b/iC3b detectable in the assay and distort data collected. However, the short timespan of this experiment means that this effect is likely to be negligible.

Initial data shown in Figure 6.21 show MFI values following incubation of three clones from each of WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ groups with Ab specific for C3b/iC3b. Deposition of C3 fragments followed a similar pattern on each cell line whereby no C3b/iC3b was detected in the absence of C. Deposition levels were observed to increase corresponding with increased serum concentration. In each case, deposition tended towards a maximal level between 2000-3000 MFI units. In most cases the three clones of each genotype behaved consistently though two clones in $CD55^{-/-}.CD59^{-/-}$ group activated C to a lesser extent than others. However, it is unclear from these data whether differences in C3b/iC3b deposition are caused by differential binding of the sensitising Ab rather than C sensitivity. The levels at which the Ab binds could be causing or masking potential differences between clones by activating C to different extents in different groups. As such, cells were assayed for binding of pAb via incubation with 5% sensitising Ab followed by detection using a donkey anti-rabbit secondary Ab conjugated to an Alexa-Fluor-594 fluorescent probe. Figure 6.22 illustrates amounts of fixing Ab bound per cell line with a clear difference observed between WT and the other groups. WT clones were shown to exhibit a significantly smaller shift in MFI than $C1q^{-/-}$ ($p=0.005$), $C3^{-/-}$ ($p=0.003$) and $CD55^{-/-}.CD59^{-/-}$ ($p=0.05$). Different levels of bound sensitising Ab is likely to skew measurement of C3 deposition

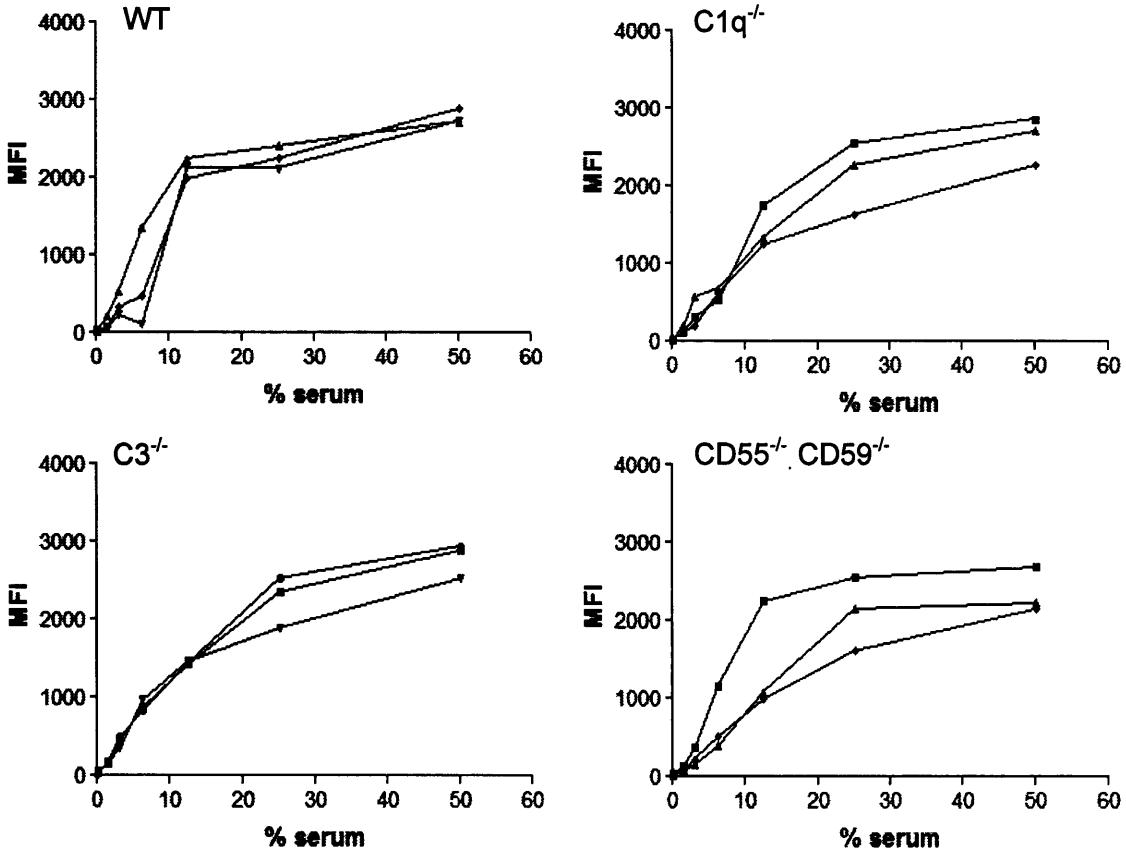
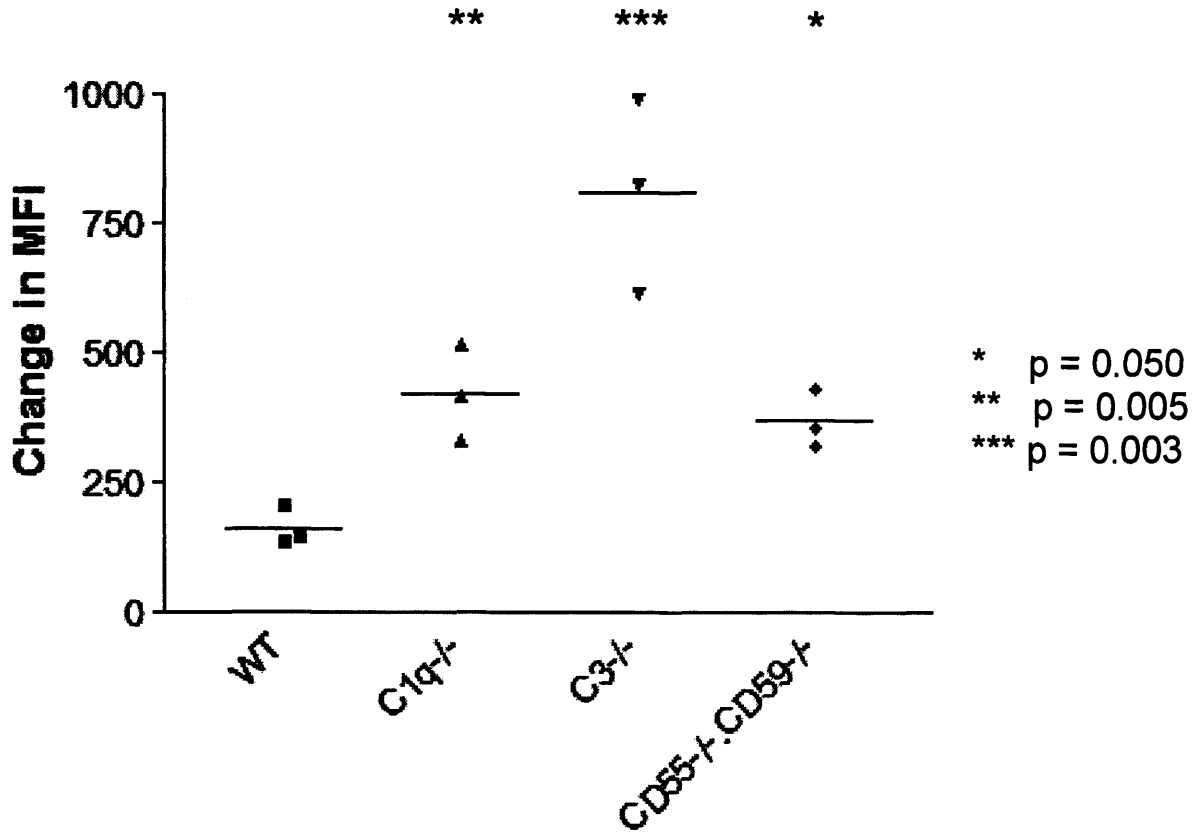


Figure 6.21: C3b deposition on clonal fibrosarcoma populations
Three clonal cell lines of each genotype were sensitised using 5% polyclonal rabbit anti-mouse erythrocyte Ab. Cells were washed before incubation with a range of mouse serum dilutions. C3b deposition was assessed via flow cytometry. Cells were stained with rat anti-mouse C3, and bound Ab visualised using RPE-conjugated donkey anti-rat IgG. Median fluorescence intensities are shown.



Genotype	Mean±SEM
WT	164±23
$C1q^{-/-}$	423±53
$C3^{-/-}$	813±107
$CD55^{-/-}.CD59^{-/-}$	372±32

Figure 6.22: Binding of sensitising Ab to fibrosarcoma populations
Clonal populations of WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ genotypes were incubated with polyclonal rabbit anti-mouse erythrocyte Ab. Bound Ab was detected using RPE-conjugated donkey anti-rabbit IgG. Control cells stained with secondary Ab only were used to calculate median shift in fluorescence. Three clones are shown. Data was analysed compared to WT using the students unpaired t-test.

in subsequent experiments and prevents accurate comparison of C3 deposition between groups.

In order to sensitise all clones to the same extent, a range of Ab dilutions was used to bind each clonal line and a sample of the population stained for presence of sensitising Ab as previously described. Figure 6.23 shows highly consistent sigmoidal curves and indicates that populations of genotypes bind pAb in different ways. However, virtually no variation was observed between clones of the same genotype. Particularly noticeable was the maximal amount of pAb bound in each group with maximum MFI shifts showing a clear trend: WT (~550) < $CD55^{-/-}.CD59^{-/-}$ (~ 650) < $C1q^{-/-}$ (~750) < $C3^{-/-}$ (~ 1000).

A change in MFI value equating to approximately 500 was selected to achieve significant but not maximal sensitisation on each clone. Selecting serum dilutions eliciting similar amounts of Ab binding allowed normalisation of the experiment and significantly reduced the effect of Ab binding as a variable. The dilution of sensitising Ab corresponding to this level of Ab fixation was selected in each clonal population, as detailed in Figure 6.24. The remaining cells were then incubated with a range of serum dilutions, before staining for C3b/iC3b as described. Differing clonal lines of the same genotype were found to require only marginally different Ab dilutions to achieve equivalent binding (WT and $CD55^{-/-}.CD59^{-/-}$ groups only showed any difference). Discrepancies between groups were noticeably larger with differing genotypes requiring differing Ab dilutions. Figure 6.25 depicts C3 deposition levels increasing according to serum concentration on clones each fixed with similar amounts of sensitising Ab. As previously, clones tended towards a plateau of maximal C3 deposition with a maximal shift of approximately 2000 MFI units observed for each clone. In order to accurately compare levels of C3b/iC3b deposition on each cell population, an estimation of Effective Concentration 50% (EC50) was made by determining concentration of serum required to cause an MFI shift of 1000 (50% of maximum). As shown in Figure 6.26 there were clear differences observed between groups while EC50 values were found to be consistent within each group. This intra-group consistency contributes to the statistically significant differences observed between WT

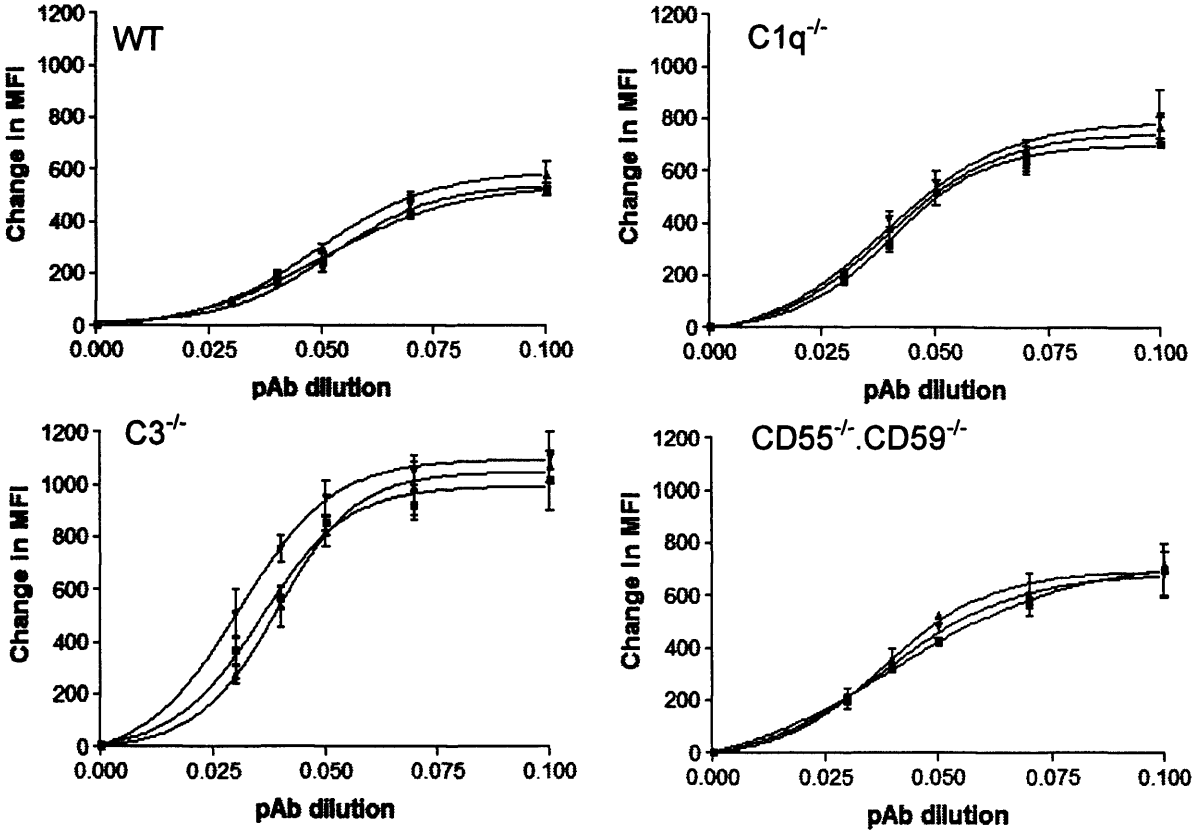


Figure 6.23: Sensitising Ab binding to clonal fibrosarcoma populations
Three clonal cell lines of each genotype were sensitised using a range of dilutions of rabbit anti-mouse erythrocyte Ab dilutions. Cells were washed before incubation with a RPE-conjugated donkey anti-rabbit IgG. Control cells stained with secondary Ab only were used to calculate mean shifts in MFI. Cells were assayed in duplicate with data from three clones shown per graph.

Cell Line	Dilution	Δ MFI
WTc	1/15	444
WTh	1/15	481
WTj	1/10	499
$C1q^{-/-}e$	1/20	469
$C1q^{-/-}i$	1/20	532
$C1q^{-/-}j$	1/20	525
$C3^{-/-}d$	1/25	529
$C3^{-/-}e$	1/25	516
$C3^{-/-}g$	1/30	500
$CD55^{-/-}.CD59^{-/-}d$	1/20	446
$CD55^{-/-}.CD59^{-/-}j$	1/20	515
$CD55^{-/-}.CD59^{-/-}k$	1/20	488

Figure 6.24: Selected dilution of sensitising Ab per clonal line
Clones were incubated with a range of dilutions of rabbit anti-mouse erythrocyte, washed and stained using an RPE-conjugated donkey anti-rat secondary antibody. Appropriate dilutions of sensitising antibody were chosen for each clone yielding an approximate shift in MFI of 500 as compared to secondary only control.

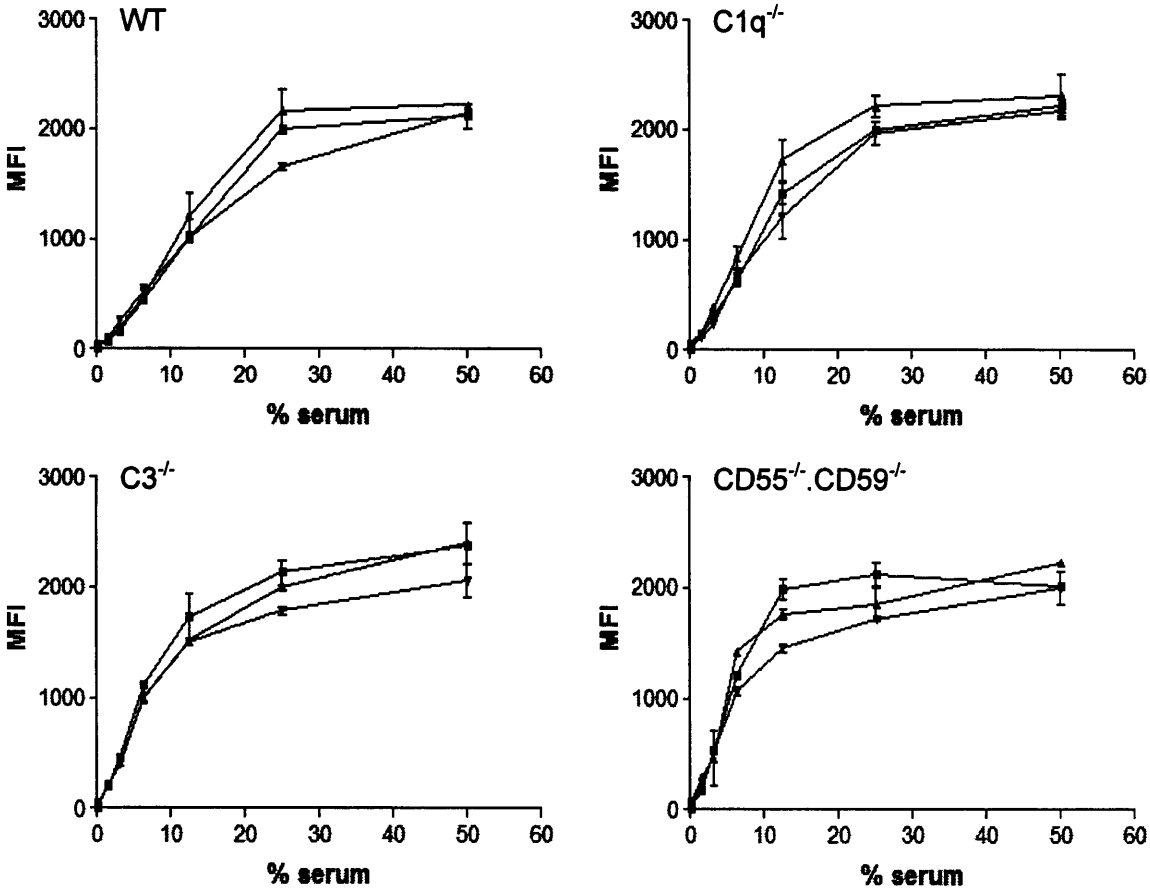
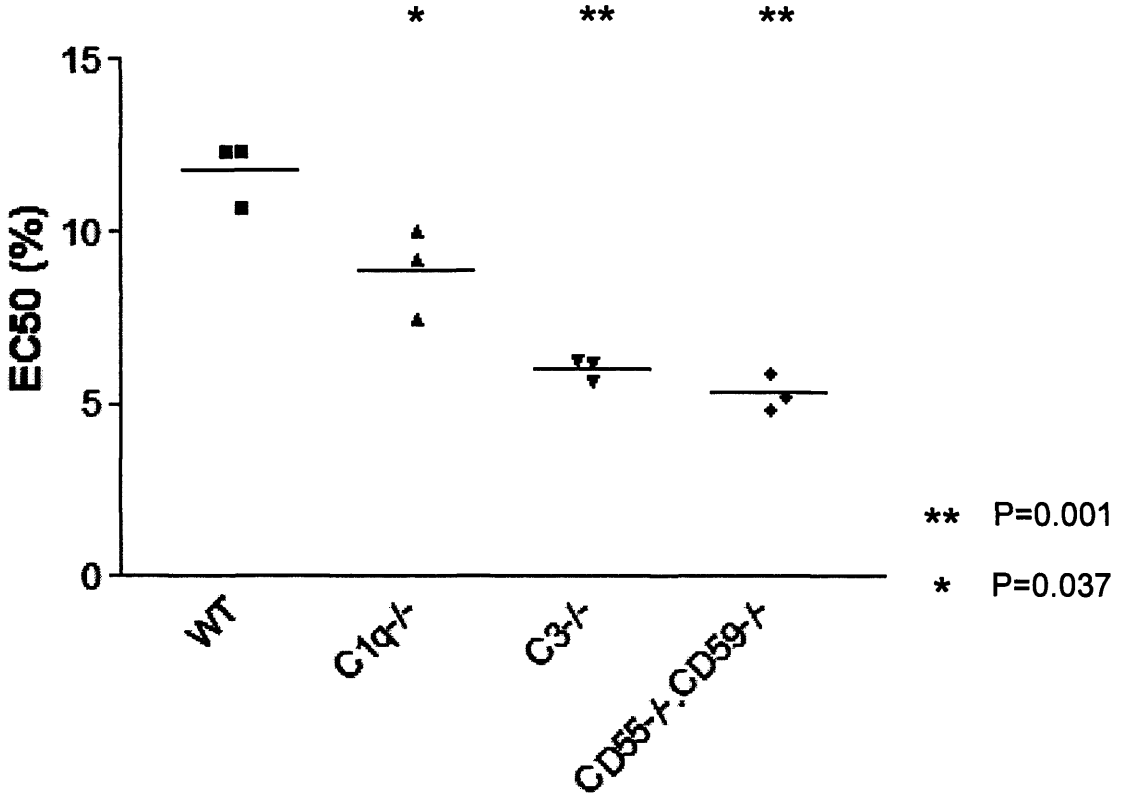


Figure 6.25: C3b deposition on populations with normalised sensitisation
Three clonal cell lines of each genotype were sensitised using rabbit anti-mouse erythrocyte pAb. Cells were washed before incubation with a range of mouse serum dilutions. C3b deposition was assessed via flow cytometry. Cells were stained with rat anti-mouse C3, and bound Ab visualised using RPE-conjugated donkey anti-rat IgG. Median fluorescence intensities are shown. Cells were assayed in duplicate with data from three clones shown per graph.



Genotype	Mean±SEM
WT	11.8 ± .0
$C1q^{-/-}$	8.9 ±1.3
$C3^{-/-}$	6.0 ±0.3
$CD55^{-/-}.CD59^{-/-}$	5.3 ±0.5

Figure 6.26: EC50 analysis of C3b deposition on fibrosarcoma cells
Dilutions of sera required to elicit half maximal C3b deposition (EC50) were calculated for each of three clones per genotype. Data shown include mean ± SEM of three clones and were analysed using the unpaired students t-test.

clones and $C1q^{-/-}$ (p=0.037), $C3^{-/-}$ (p=0.001) and $CD55^{-/-}.CD59^{-/-}$ (p=0.001) with WT requiring a significantly higher dose than each other group to evoke the same level of C3 deposition. Deficiency in CD55.CD59 appears to render populations more susceptible to C3 deposition than WT counterpart, as would be expected due to impaired C regulatory mechanisms. However, reasons underpinning the increased susceptibility of C1q and C3 deficiencies are less obvious yet remain significant.

6.7 *in vivo* growth of selected fibrosarcoma clones

Key experiments planned for this chapter involved investigation of the incidence / rate of tumour growth *in vivo* exhibited by tumour cell lines with and without the ability to synthesise C1q, C3 or express the CRegs CD55 and CD59. However, due to time constraints, a full investigation utilising these cell lines was not possible. A preliminary analysis of a potential role for C3 synthesis and secretion by tumour cells *in vivo* was performed. Data shown previously (Figures 6.11 and 6.14) suggests that WT cells do not secrete or synthesise C3 *in vitro*. However, cells derived from a WT animal possess the C3 gene, and may be stimulated to secrete C3 into the tumour micro-environment *in vivo*. In contrast, $C3^{-/-}$ tumours do not have the gene encoding C3 and are unable to synthesise the protein. Three WT and three $C3^{-/-}$ lines were selected which exhibited similar growth kinetics *in vitro* (as shown in Figure 6.6). One observed difference between WT and $C3^{-/-}$ lines is the consistent expression of CD59 on $C3^{-/-}$ clones (illustrated in Figure 6.8). This is expected to confer enhanced protection from MAC deposition and lysis to these cells compared to WT cells lacking CD59 while immunological roles for the CD59 protein may also be significant. Re-inoculation of these cells back into WT and $C3^{-/-}$ host animals has the potential to elucidate differences in C susceptibility *in vivo* and allow determination of the source of any C3 deposited on these tumours i.e. whether C3 detected via immunohistochemical staining is derived from host immune system or locally produced by tumour cells (see Table 6.1).

Clonal fibrosarcoma lines were generated and selected as described above. The three clonal lines of WT and $C3^{-/-}$ genotype were re-inoculated into groups of 4 WT or $C3^{-/-}$ as previously described in Chapter 4. However, the majority of mice did not develop a tumour. Several tumours were observed but arose later than expected and exhibited atypical growth characteristics. Figure 6.27 illustrates poor tumour incidence in WT animals of three WT and three $C3^{-/-}$ clonal lines. Additionally, the K6 fibrosarcoma line was included and also found to exhibit poor tumour take. These lines were injected simultaneously into $C3^{-/-}$ hosts with similarly poor tumour incidence observed. This suggests that the lack of growth is not caused by CDC or C3-mediated opsonisation. As previously described in Chapters 3 and 4, the K6 tumour line is known to have high incidence and grow aggressively in both WT and $C3^{-/-}$ mice. However, these experiments were performed approximately 18 months after the observations in Chapter 4 and yielded markedly different results with <40% tumour incidence observed, compared to ~90% in Chapter 4. Despite repeated attempts to achieve typical tumour incidence following K6 inoculation, mice appeared resistant to tumour growth and incidence remained low. Figure 6.27 incorporates collated data from several separate experiments and illustrates the poor growth rates observed following inoculation of each clonal line. There is insufficient tumour incidence to form any meaningful conclusions from these data. It appears that external factors were preventing tumour incidence and distorting observations. Although K6 tumour growth remained inconsistent and with uncharacteristically low incidence, 9 out of 24 (WT and $C3^{-/-}$ combined) mice did develop a tumour. This is a higher proportion than any other clonal line, the next highest incidence was observed in a WT clone (3 out of 24).

The inability to grow tumours at this time corresponded with the commencement of building works close to the animal housing facility. It is possible that raised stress levels and an altered immune response contributed to the irregular tumour growth observed. Whilst numerous factors are likely to impact on immune responses, noise levels have been linked to immunomodulation of T-lymphocytes (Monjan et al. 1977), and macrophages (Spehner et al. 1996). The finding that growth was not restored in $C3^{-/-}$ host

WT host	WTc	WTh	WTj	C3d	C3e	C3g	K6
Mice inoculated	16	12	12	8	8	8	16
Mice developing tumour	2	1	0	1	0	0	6

$C3^{-/-}$ host	WTc	WTh	WTj	C3d	C3e	C3g	K6
Mice inoculated	8	8	4	4	4	4	8
Mice developing tumour	1	0	0	0	0	0	3

Figure 6.27: Tumour incidences following inoculation of mice with fibrosarcoma cells
Cells were inoculated in 100μL PBS sub-cutaneously on the mouse flank. Male WT and $C3^{-/-}$ mice were inoculated with 10^5 cells of various WT and $C3^{-/-}$ clonal fibrosarcoma populations. Number of mice developing a palpable tumour is indicated. Data is pooled from 3 separate experiments.

animals suggests that the effect is unlikely to be mediated by C whereby only the very early stages of CP or MBL pathway activation are possible. In order to ensure that cells were free of mycoplasma and endotoxin contamination, all populations were screened and found to be mycoplasma negative and endotoxin-low. As an additional control, K6 cells were inoculated into $Rag^{-/-}$ mice simultaneously with WT mice. $Rag^{-/-}$ mice possess no mature B-cells or T-cells and thus exhibit severely impaired immune responses (Mombaerts et al. 1992). These data are shown in Figure 6.28 and illustrate rapid tumour growth and 100% incidence in $Rag^{-/-}$ mice while WT incidence remained low. This observation suggests that the cells are able to grow normally without immune pressures (B-cell/T-cell responses) and further suggests a link between immune responses and tumour clearance in this model.

Following a period where tumour growth was of low incidence and erratic, growth of K6 populations in WT mice was observed to be close to normal levels (6 mice developing tumours from 8 inoculated). Subsequently, heterogeneous (parent) fibrosarcoma populations were inoculated into WT and $C3^{-/-}$ male mice and tumour growth monitored. These lines were expected to provide a more robust model for examination of growth characteristics due to positive selection of cells most suitable for *in vivo* growth i.e. least immunogenic. Two WT and two $C3^{-/-}$ non-clonal populations were inoculated into both WT and $C3^{-/-}$ hosts. Tumour volumes were measured over a one month period following inoculation and are displayed in Figure 6.29. As can be seen, tumour incidence was higher than previously recorded (88% incidence in pooled data). Growth curves exhibited the conventional exponential characteristic and suggested that tumour growth had returned to previously observed and consistent kinetics. Few comparisons can be made from data of this type, however, a visual trend was identified whereby $C3^{-/-}$ cells formed a palpable tumour in WT mice (Panel B) more rapidly than the same cells inoculated into $C3^{-/-}$ host mice (Panel D) i.e. tumours arose at an earlier timepoint in WT mice. However, incidence data displayed in Figure 6.30 do not support this observation and while a trend to this effect is observed, the p-value of 0.643 indicates that the effect is not statistically significant following analysis using the log-rank statistical test. Corresponding

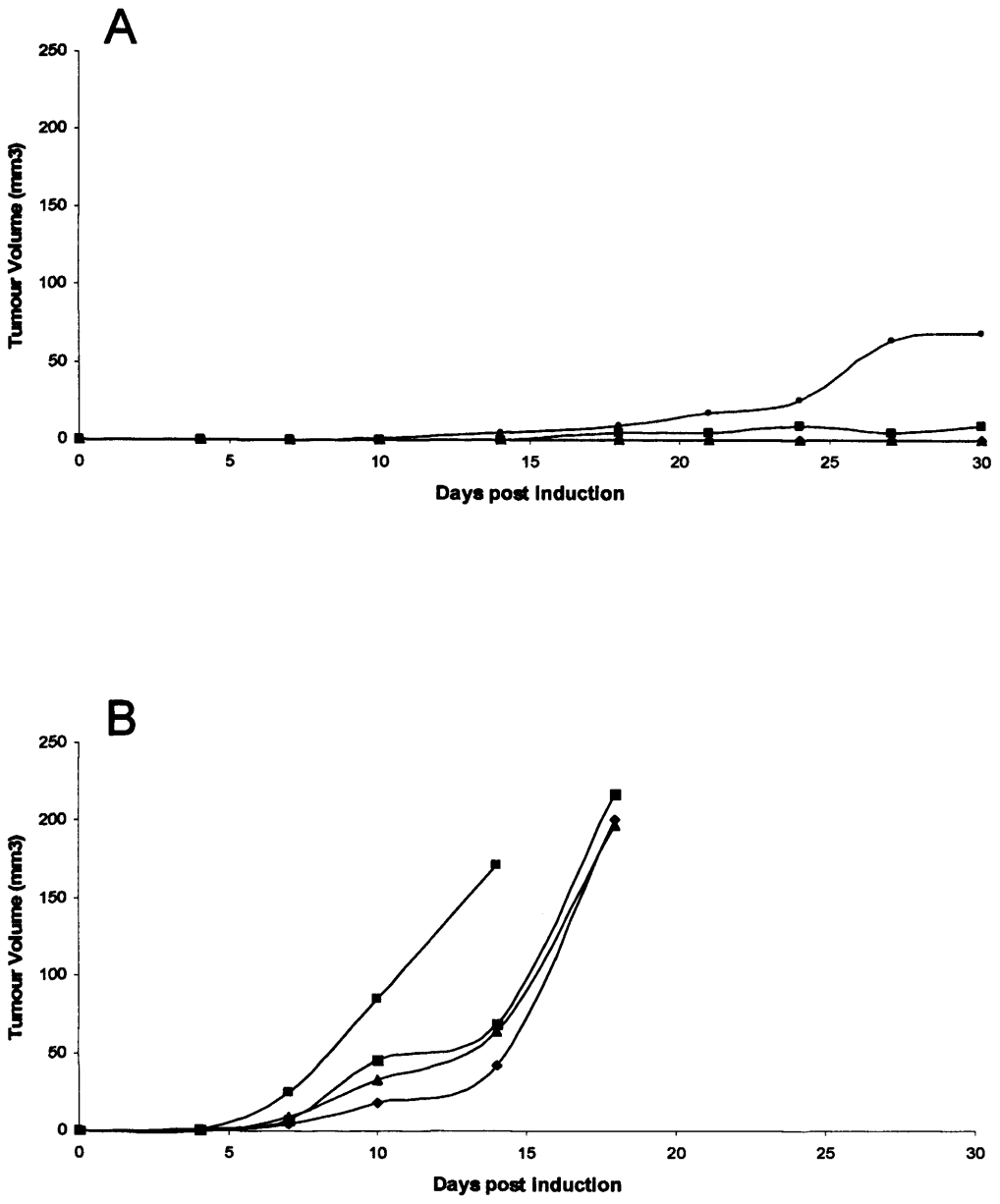


Figure 6.28: Inoculation of K6 cells into WT and Rag$^{-/-}$ male mice
Inoculation of 10^5 K6 cells into WT (panel A) and Rag$^{-/-}$ (panel B) mice. Mice were injected on day 1 with 100µL tumour cells on the flank and monitored regularly with tumour sizes measured. Each line is representative of one individual mouse. Mice were sacrificed when tumour reached 200mm^3. Each group consists of 4 mice.

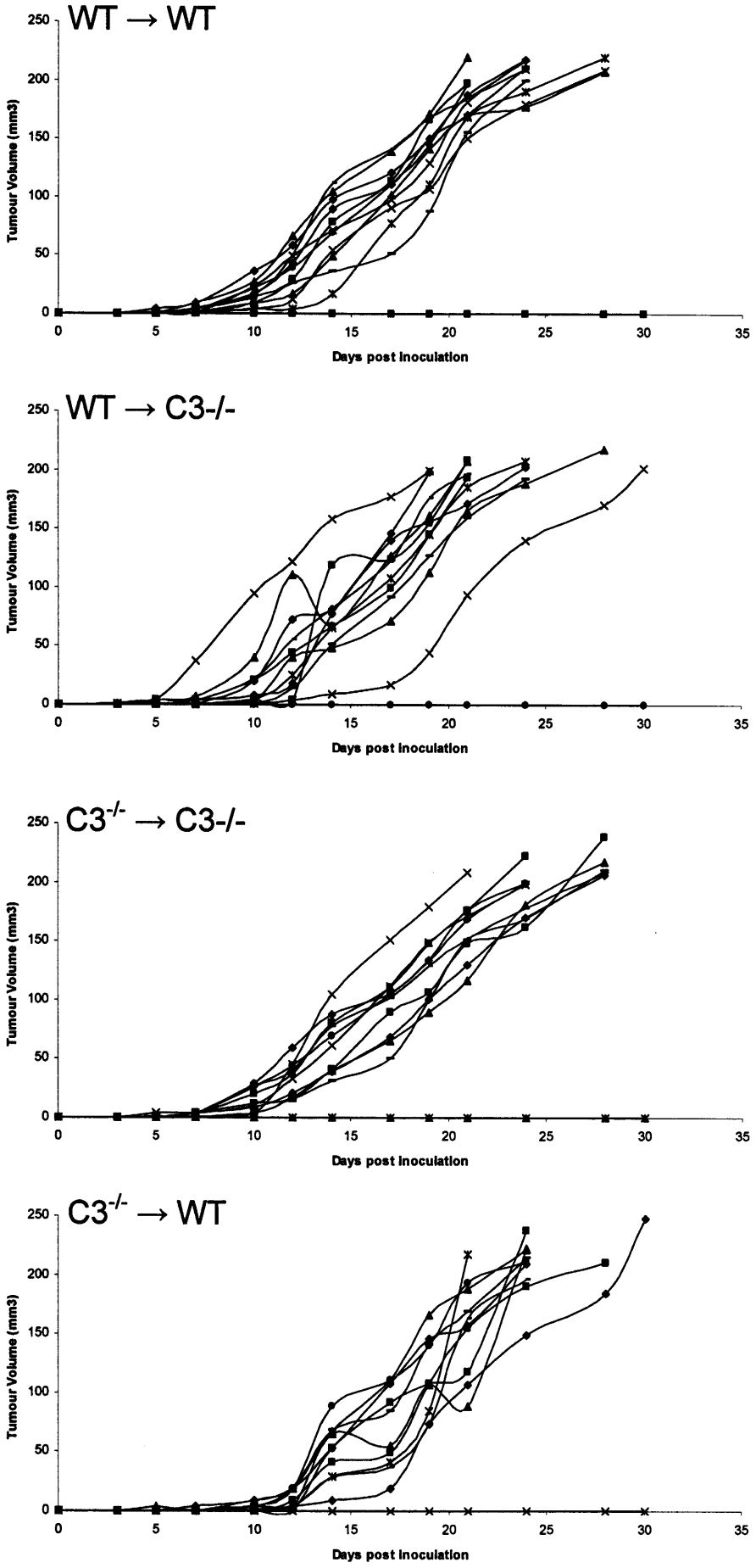


Figure 6.29: Inoculation of heterogeneous fibrosarcoma populations into WT and $C3^{-/-}$ mice
Inoculation of 10^5 heterogeneous cells into male mice. Mice were injected on day 1 with 100μL tumour cells on the flank and monitored regularly with tumour sizes measured. Each line is representative of one individual mouse. Mice were sacrificed when tumour reached $200mm^3$.

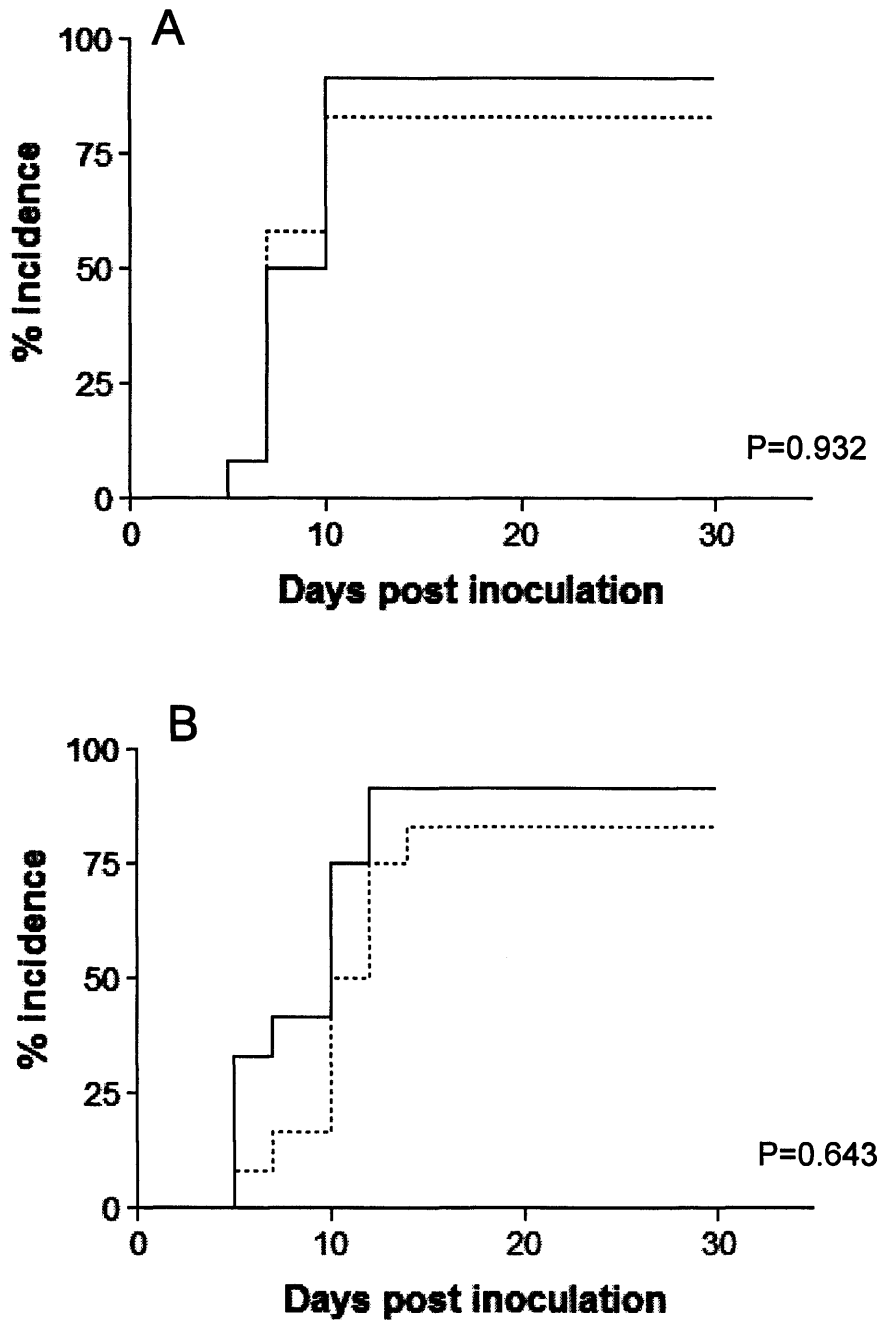


Figure 6.30: Tumour incidence in WT and $C3^{-/-}$ mice
Tumour cells were inoculated into WT (bold lines) and $C3^{-/-}$ male (dashed lines) mice and tumour incidences noted. Inoculation of WT cells (graph A) and $C3^{-/-}$ cells (graph B) are shown. Data were analysed using the logrank statistical test.

data showing incidence of WT tumours in WT and $C3^{-/-}$ mice revealed no difference between the groups (p=0.932). Survival data derived from the same tumour volume measurements is shown in Figure 6.31 and suggested a weak trend towards enhanced tumour growth in WT mice compared to $C3^{-/-}$. However, this trend is not statistically significant following inoculation of either WT (p=0.215) or $C3^{-/-}$ cells (p=0.159). Data were further analysed for differences in WT tumour incidence and survival in WT and $C3^{-/-}$ hosts. These data are shown in Figures 6.32 and 6.33 and found no difference in terms of incidence (p=0.832) or survival (p=0.502). Corresponding data following inoculation of $C3^{-/-}$ is also included on the above Figures with similar findings observed for incidence (p=0.264) and survival (0.671).

Excised tumours were sectioned and stained for intracellular C3 stores or C3b/iC3b deposition by immunohistochemistry (Figure 6.34). Following quantitation of these data, comparable deposition was observed on both WT and $C3^{-/-}$ tumour cells inoculated into WT hosts (Figure 6.35). Conversely, no C3/C3b/iC3b presence was detected on the same WT and $C3^{-/-}$ cell lines inoculated into $C3^{-/-}$ hosts. This observation strongly suggests that C deposition observed on these cells is derived from the host and is not tumour derived as evidenced by the lack of deposition on WT cells in a $C3^{-/-}$ host. This is consistent with the *in vitro* lack of secreted C3 or intracellular C3 mRNA described above. It is possible that tumour cells do secrete C3 or activation products but that an initial nidus of C3b deposition is required to kick-start this mechanism. However, a more likely conclusion from data described in this Chapter is that neither WT nor $C3^{-/-}$ fibrosarcoma cells generated in this model synthesise or secrete C3 *in vitro* or *in vivo*.

Further immunohistochemical analysis was performed by staining for the presence of C9 in these tissues (Figures 6.36 and 6.37), providing a marker for terminal pathway activation and MAC deposition. These data closely resembled the previous findings for deposition of C3b/iC3b with significant levels of C9 present in WT hosts, but none observed in $C3^{-/-}$ hosts further supporting the findings above and also providing evidence against C9 synthesis by the fibrosarcoma cells since $C3^{-/-}$ cells cannot generate C9 by C

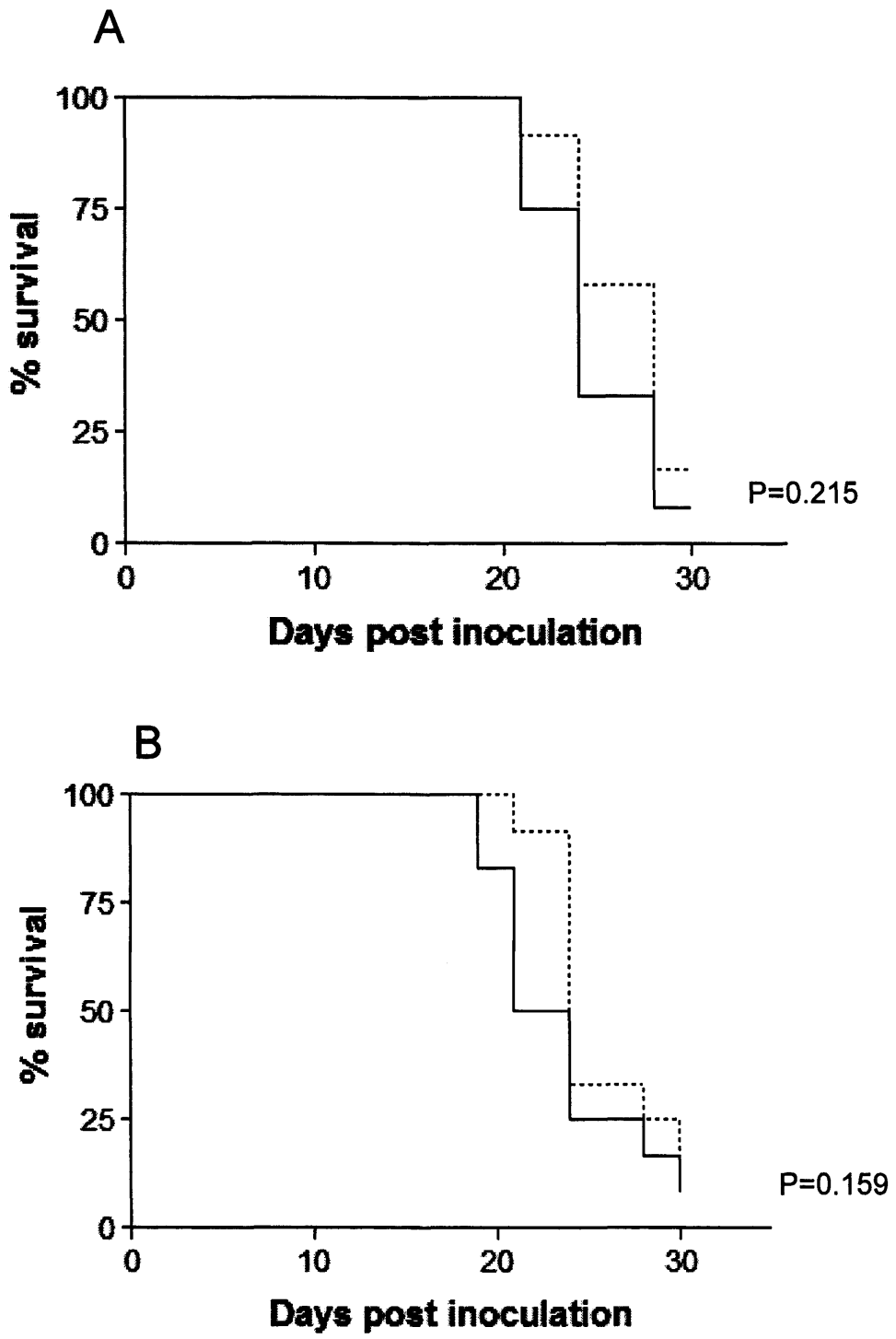


Figure 6.31: Percentage survival in WT and $C3^{-/-}$ mice
Tumour cells were inoculated into WT (bold lines) and $C3^{-/-}$ (dashed lines) male mice and survival data noted. Inoculation of WT cells (graph A) and C3-/- (graph B) cells are shown. Data were analysed using the logrank statistical test.

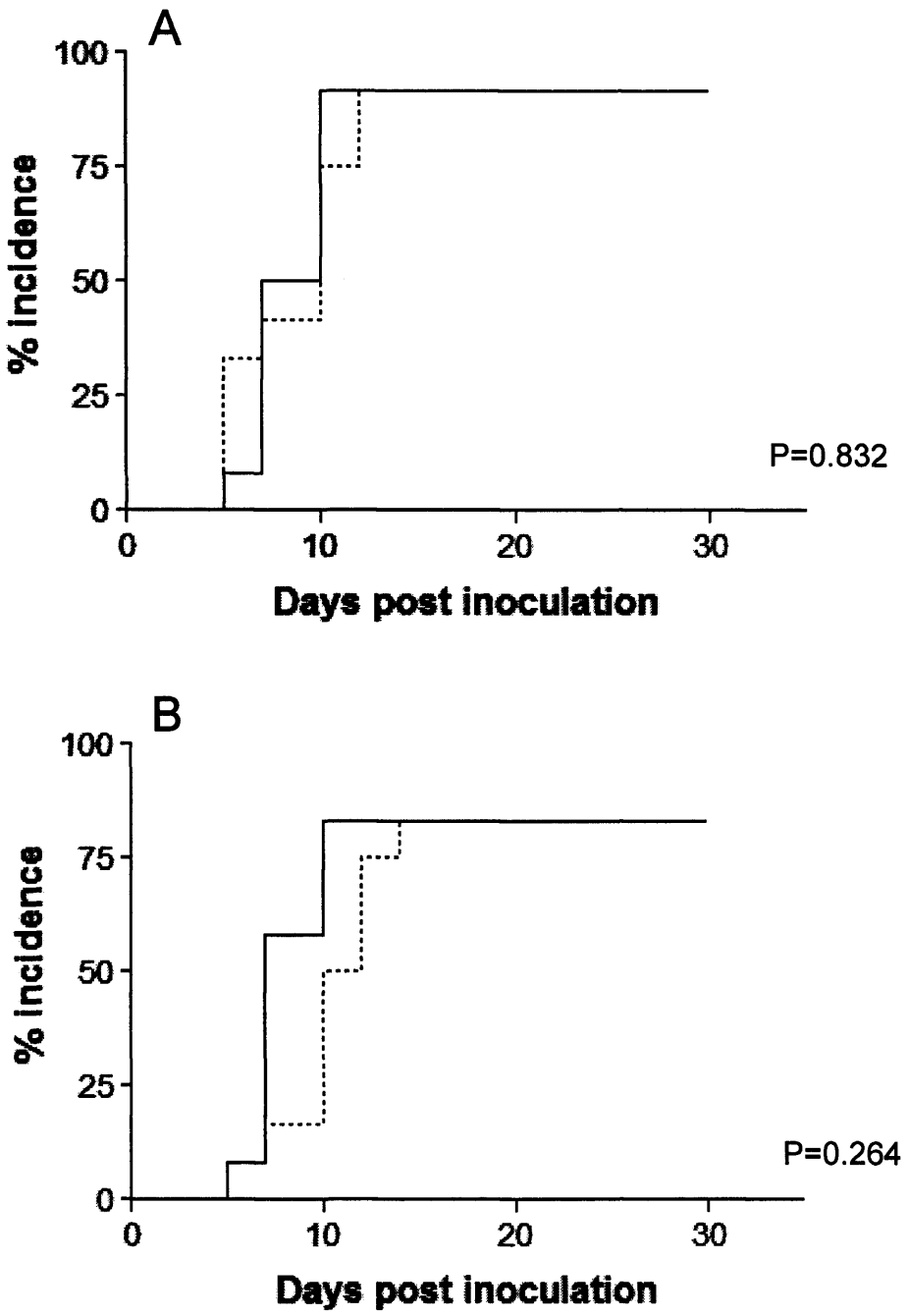


Figure 6.32: Tumour incidence in WT and $C3^{-/-}$ mice
Tumour cells were inoculated into WT (graph A) and $C3^{-/-}$ male (graph B) mice and tumour incidences noted. Inoculation of WT cells (bold lines) and $C3^{-/-}$ cells (dashed lines) are shown. Data were analysed using the logrank statistical test.

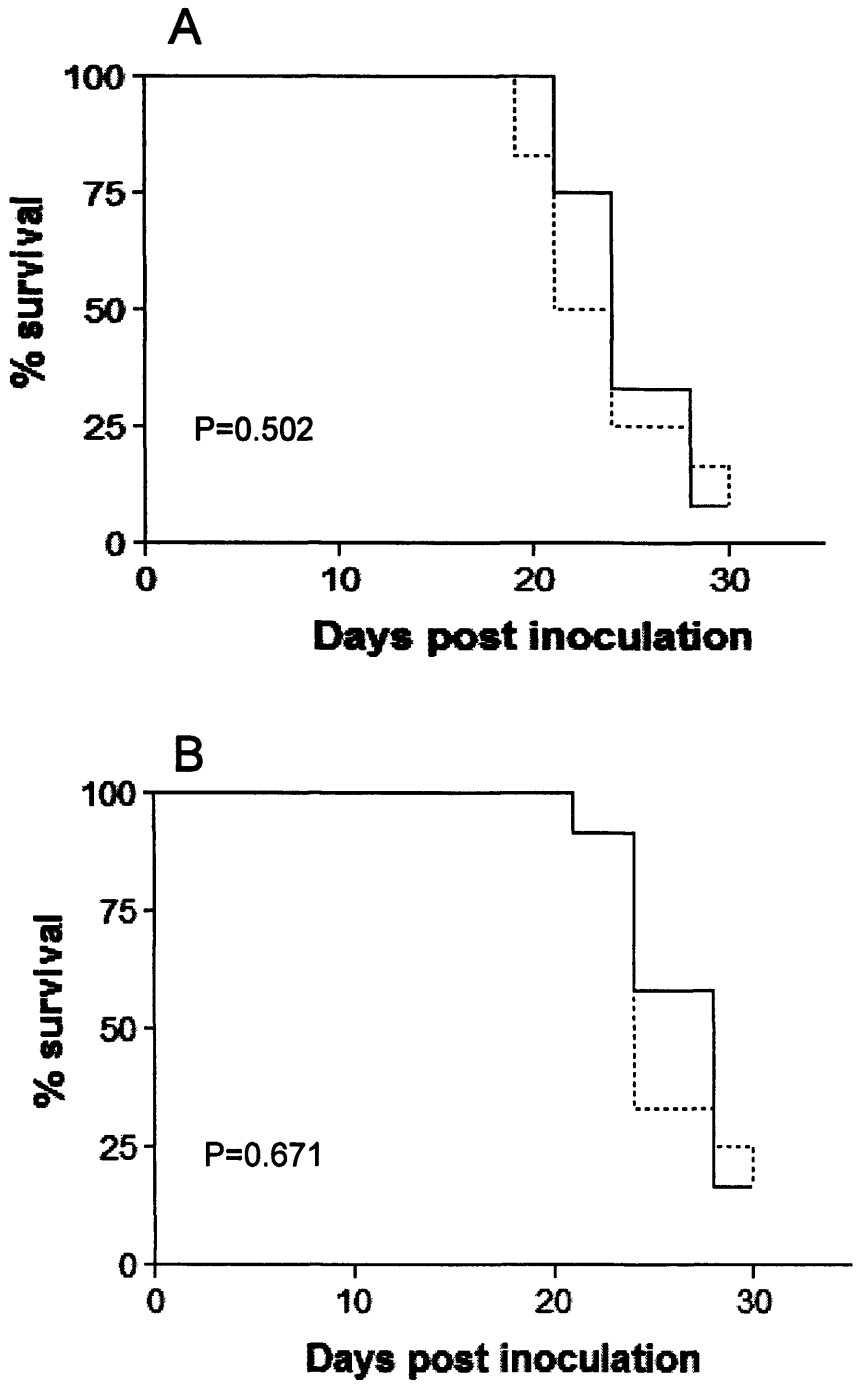


Figure 6.33: Percentage survival in WT and $C3^{-/-}$ mice
Tumour cells were inoculated into WT (graph A) and $C3^{-/-}$ (graph B) male mice and survival data noted. Inoculation of WT cells (bold lines) and C3-/- cells (dashed lines) are shown. Data were analysed using the logrank statistical test.

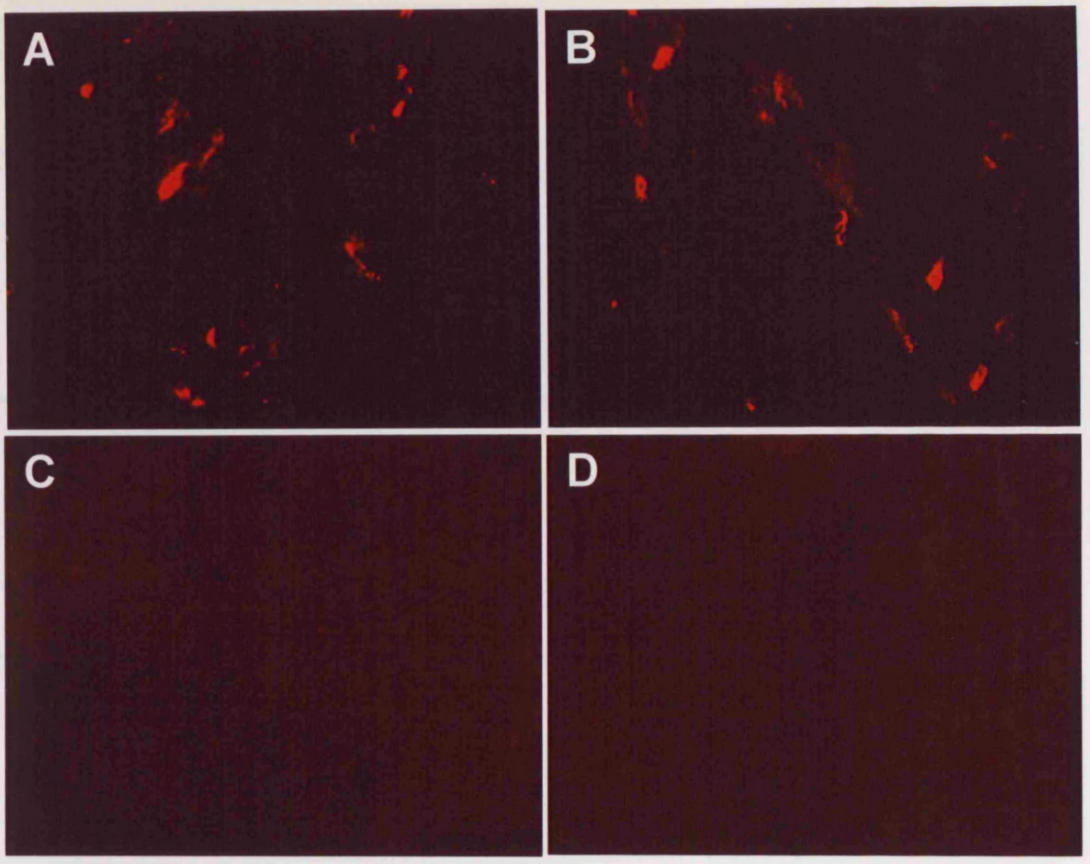
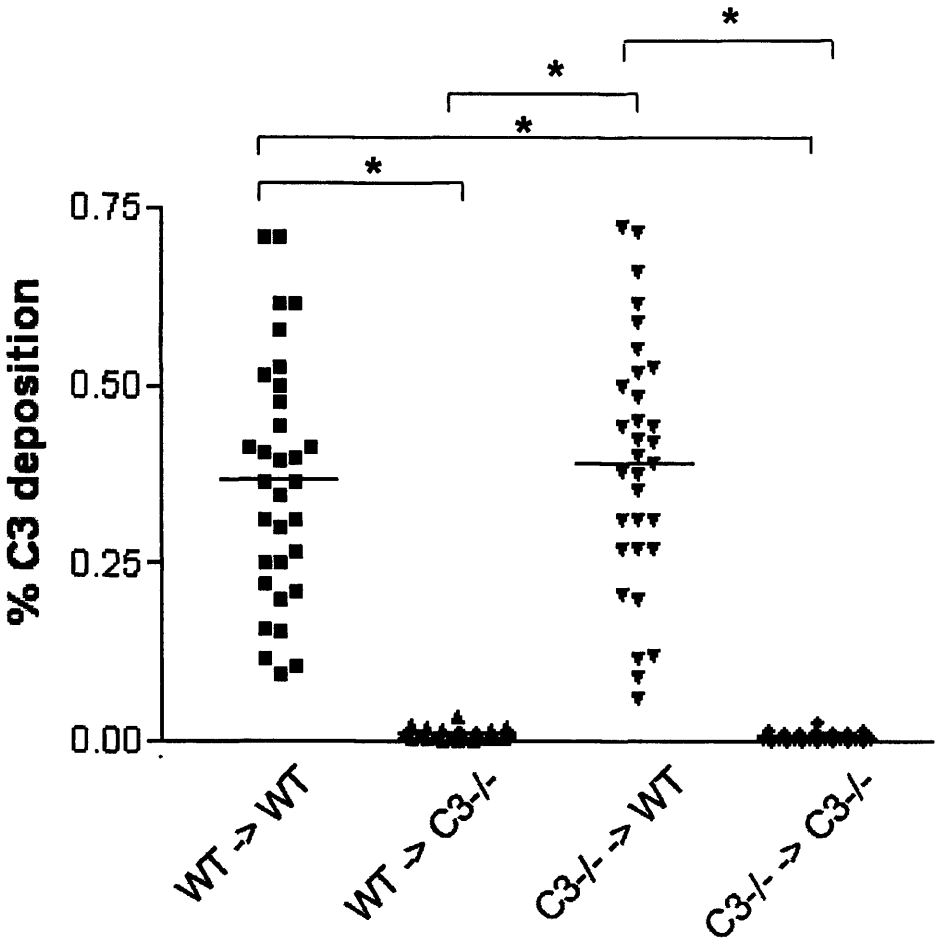


Figure 6.34: C3 deposition on heterogeneous fibrosarcoma populations
Heterogeneous WT cell populations were inoculated into WT (A) and $C3^{-/-}$ (C) male mice. Heterogeneous $C3^{-/-}$ cell populations were inoculated into WT (B) and $C3^{-/-}$ (D) male mice. Excised tumours were sectioned and stained for C3/C3b/iC3b presence by incubation with a rat anti-mouse C3 mAb and detected using Alexa Fluor-594 conjugated donkey anti-rat IgG secondary Ab. Panels shown are representative of 8 sections per tumour, from 4 separate tumours per group.



	Mean ± SEM
WT→ WT	0.367 ± 0.030
WT→ $C3^{-/-}$	0.010 ± 0.001
$C3^{-/-}$→ WT	0.391 ± 0.031
$C3^{-/-}$→ $C3^{-/-}$	0.007 ± 0.001

Figure 6.35. Immunohistochemical analysis of C3b/iC3b deposition
Percentage area stained positive for C3b/iC3b deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 4 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test. * indicates p-value <0.05.

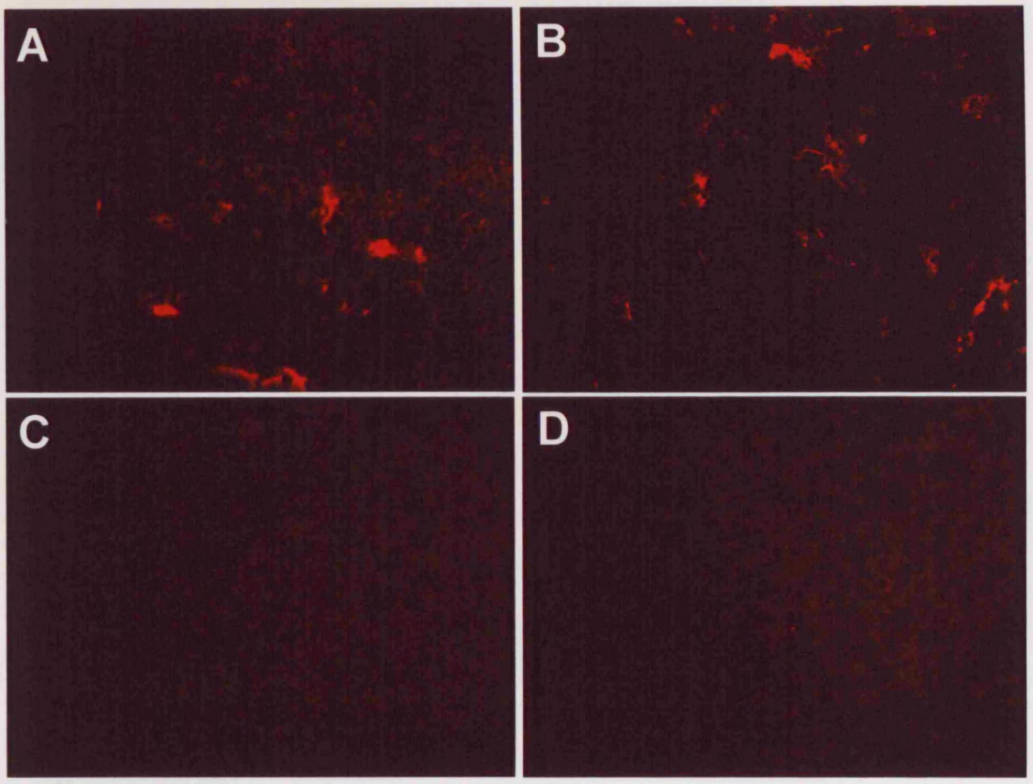
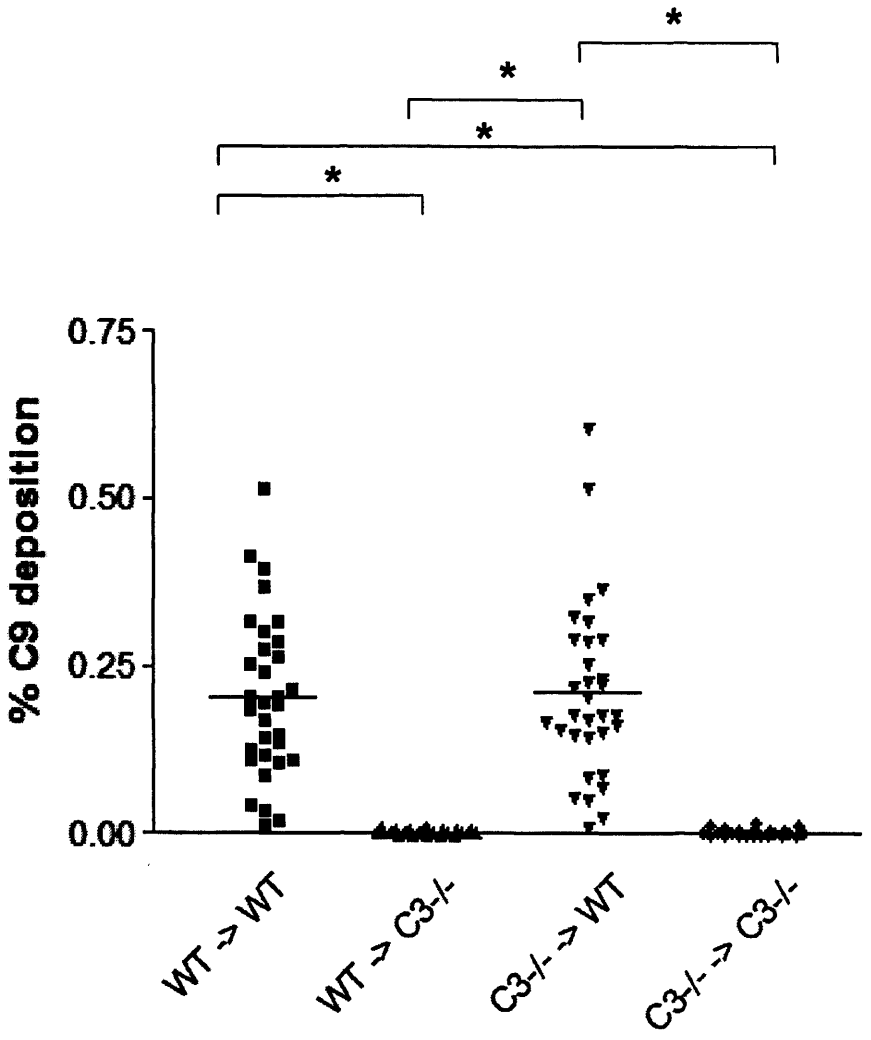


Figure 6.36: C9 deposition on heterogeneous fibrosarcoma populations
Heterogeneous WT cell populations were inoculated into WT (A) and $C3^{-/-}$ (C) male mice. Heterogeneous $C3^{-/-}$ cell populations were inoculated into WT (B) and $C3^{-/-}$ (D) male mice. Excised tumours were sectioned and stained for C9 presence via incubation with rabbit anti-rat C9 and Alexa-fluor-594 conjugated goat anti-rabbit IgG. Panels shown are representative of 8 sections per tumour, from 4 separate tumours per group.



	Mean ± SEM
WT→ WT	0.204 ± 0.021
WT→ $C3^{-/-}$	0.003 ± 0.000
$C3^{-/-}$→ WT	0.211 ± 0.023
$C3^{-/-}$→ $C3^{-/-}$	0.003 ± 0.001

Figure 6.37. Immunohistochemical analysis of C9 deposition
Percentage area stained positive for C9 deposition was assessed using Image Pro Analyser software, 8 frames were assessed per tumour with 4 tumours per group used. Data were analysed using a 2 tailed, unpaired t-test. * indicates p-value <0.05.

activation, but fibrosarcoma cells have been shown to synthesise and secrete C9 (Garred et al. 1990).

6.8. Discussion

Clonal fibrosarcoma lines were generated in order to provide homogeneous populations with consistent and reproducible characteristics both *in vitro* and *in vivo*. The cloning procedure used gave rise to uniform populations in terms of CD140a expression. This molecule represents a useful marker for fibrosarcoma phenotype and was expressed in all cell lines analysed despite reported down-modulation of such antigens following 3-MCA tumour induction (Kopp et al. 1995, Schreier et al. 1988).

It was considered necessary to generate homogeneous populations in order to attain consistent *in vitro* characteristics such as proliferation rate and C-susceptibility and to allow comparison between lines. This allowed selection of lines which behave consistently and are therefore more likely to yield reliable results. Expression of CReg was also shown to be consistent between clones of the same genotype.

Therefore, clonal lines of each genotype (WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$) were assayed for *in vitro* proliferation rates. The use of Alamar Blue as a measure for proliferation provided a reproducible comparator of growth kinetics on cell populations. From these data, lines exhibiting anomalous growth curves were discarded and 3 comparable clones selected from each group. Data displayed on the proliferation graphs were consistent with expected growth patterns showing a lag, log and stationary phase. Each cell line was tested in triplicate and data shown are representative of 2 separate experiments. The lack of variation observed within the same line further supports the use of the Alamar Blue proliferation assay for this purpose. Three clones displaying consistent and 'average' growth patterns were selected from each group, clones deficient in C1q-/-, C3-/- and CD55-/-.CD59-/- were then re-analysed and compared to clones from the WT group. All 12

selected clones grew comparably and reproducibly *in vitro* when re-analysed in the same experiment at the same time (Figure 6.6).

All 12 clones were shown to express high levels of Crry, this is consistent with post-excision staining of MCA-induced tumours as shown in Chapter 5. This molecule is ubiquitously expressed by murine cells and has been shown to enhance tumour growth in a rat breast cancer model (Caragine et al. 2002). Additionally, enhanced anti-tumour responses have been observed following blockade of Crry (Ohta et al. 2004, Gelderman et al. 2004). Significant expression of Crry is expected on these tumour lines and provides an effective control mechanism against early stage C deposition and C-mediated lysis. Despite being generated in a host lacking a functional C system and expressing CD59, $C3^{-/-}$ cells were shown to express comparable levels of Crry to those generated from other genotypes.

Lack of CD55 expression was also consistent across all 12 clones analysed with no protein detected on cell surfaces. Mouse erythrocytes were used to control for binding of MD1, a rat monoclonal anti-mouse CD55 Ab and were found to exhibit a significant shift in fluorescence. The lack of CD55 expression observed is consistent with data described in Chapter 5 whereby no CD55 expression was observed by post-excision analysis of fibrosarcoma populations. This protein is also known as 'Decay Accelerator Factor' (DAF) and serves to enhance the rate of C3 convertase decay. The complete lack of CD55 expression on fibrosarcoma cells examined in this thesis is an interesting observation given the propensity of many tumour cell types to over-express CRegs. However, the observed presence of the murine specific CReg Crry may be significant with this protein functioning in a similar fashion to CD55. Thus, CD55 may be of more relevance in human tumour models than in mice.

A clear difference in CD59 expression was observed with $C3^{-/-}$ lines shown to express this terminal pathway CReg. Cells of WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ genotypes did not express any CD59. As described in Chapter 5, WT, $C1q^{-/-}$ and $C3^{-/-}$ tumours do express low levels of CD59 *in vivo*. However, this

expression was not conserved in the three clonal lines selected from WT and $C1q^{-/-}$ tumours and indicates that CD59 expression is lost by these cell lines following culture *in vitro*. The observation that CD59 expression was not lost in $C3^{-/-}$ lines, and was conserved in all three clones, may suggest a more important role for this protein on $C3^{-/-}$ tumours. This is unexpected since host mice were lacking in C3 and have no capacity to activate the alternative or terminal pathway with only the initial activation steps of the classical pathway possible. Tumour populations generated in $C3^{-/-}$ mice did not stimulate MAC deposition (whereas MAC was deposited on WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ tissue), yet these $C3^{-/-}$ tumours were the only populations shown to conserve CD59 expression following *in vitro* culture and cloning. A number of roles for the CD59 protein outside of C regulation have been reported. These include modulation of T-cell (Deckert et al. 1995), NK-cell (Omidvar et al. 2006) and neutrophil (van den Berg et al. 1995) responses. Such roles are described in detail in Chapter 1.7.2 and are generally attributed to CD59 expression on the immune cells rather than targets although further investigation is necessary to determine whether conserved CD59 expression by $C3^{-/-}$ fibrosarcoma cells is related to the lack of C3b and MAC deposition during generation of the parent populations *in vivo*.

These data were supported by investigation of mRNA levels for CD55 and CD59 in fibrosarcoma populations. Only hepatoctyes were found to contain CD55 message, while hepatocytes and $C3^{-/-}$ populations were shown to contain CD59 message in both clonal ($p=0.007$) and heterogeneous lines ($p=0.040$). These findings support flow cytometry data and appear to rule out the possibility of CReg being stored intra-cellularly (Rowan et al. 1998) (Hatanaka et al. 1998), released in soluble form (Jurianz et al. 2001), or secreted on tumour-released exosomes *in vitro* (Clayton et al. 2003). *in vitro* characteristics were conserved in the clonal lines and consistent with those of the parent populations in each case for each of the parameters assessed in this Chapter.

Fibroblastic cells have been reported to synthesise C3 *in vitro* (Katz et al. 1989). The *in vitro* secretion of C3 by malignant melanoma cells has also

been reported with 350ng detectable by ELISA following incubation of s/n with 10^6 cells over a 24 hour period (Jean et al. 1997). However no C3 secretion, or presence of activation products was detected in culture medium incubated with any of the clonal or parent lines tested in this Chapter. A concentration of 15ng/ml and 20ng/ml (in 100µL) purified C3 was detectable in assays shown in Figures 6.11 and 6.13. The observation that s/n contained no detectable C3/C3b/iC3b following 10-fold concentration suggests that the cells are not secreting C3 *in vitro*. These data alone are not sufficient to determine the capacity of these cells to secrete C3. Whilst the cells tested may not synthesise or secrete this protein into s/n, it is possible that C3 is maintained in intra-cellular stores ready for secretion following an *in vivo* stimulus. Alternatively, C3 may only be synthesised following Interleukin stimuli such as IL-1 (Katz and Strunk 1989), IL-6 (Katz et al. 1989) and IL-13(Katz et al. 1995) as previously reported in fibroblasts. The lack of C3 message as assessed by RT-PCR appears to rule out the possibility that C3 is stored intra-cellularly *in vitro*. This technique is regarded to be highly sensitive although negligible C3 mRNA was detected in any of the clones tested. The significant amounts of C3 message found in liver cells confirms the ability of this technique to detect C3 message and that hepatocytes synthesise C3.

Susceptibility to C attack may be a key characteristic relating to the behaviour of these cell lines in an *in vivo* setting. Whilst data in Chapter 5 illustrate evidence of C activation and targeting to tumour cells, it is extremely difficult to assess contributions of C to killing *in vivo.* However, the *in vitro* sensitivity of these clones provides a useful indicator of likely scale of C attack *in vivo*. Whilst tumour cells generated in this model have effective basal resistance to C (through expression of Crry), investigation of C-susceptibility in different lines can provide clues as to possible induced mechanisms of C resistance. These cellular activation mechanisms are described fully in Section 1.12 and may be present in one or more of the genotypes generated in this model. Such mechanisms are not well understood, however, activation of a protein kinase pathway has been shown to lead to Ca^{2+} influx (Morgan and Campbell 1985), protein activation (Kraus, Seger and Fishelson 2001) and increased protein (Reiter, Ciobotariu and Fishelson 1992) and lipid (Ohanian and

Schlager 1981) synthesis following C deposition. These cellular activation events may contribute to increased proliferation or enhanced resistance to further C attack.

A potent obstacle to tumour immunotherapy is the poor C activating capacity of the majority of anti-tumour antibodies (Wang and Weiner 2008). In addition, nucleated cells are adept at removing bound Ab from their surfaces either by internalisation (Mariani et al. 1989), or shedding (Campbell, 1985). This represents an effective mechanism for removal of Ab from tumour cell surfaces and thus reduces the effects of Ab-dependent cellular cytotoxicity and C activation through CP. In order to elicit a substantial C attack on the cloned fibrosarcoma lines, a polyclonal rabbit anti-mouse erythrocyte Ab was used. This Ab binds a multitude of mouse antigens shared by erythrocytes and tumour cells as evidenced by the considerable shift in fluorescence observed on all tumour types in Figure 6.16. However, despite high titres of C-fixing Ab, negligible killing was observed by PI staining of DNA in lysed cells. Neat mouse serum was unable to cause significant cell death in tumour cells despite being shown to readily lyse sensitised erythrocytes. Nucleated cells are known to be significantly more resistant to CDC (Ohanian, 1981). However, the complete lack of lysis observed may be indicative of enhanced C-resistance of tumour cells over non-transformed cells. This could be tested by the use of non-tumour nucleated cells e.g. fibroblasts. One problem associated with this technique was the repeated settling and re-suspending of cells, leading to aggregation and consequently limiting tumour cell surface available for deposition. A rat monoclonal anti-mouse Epcam Ab against a common tumour antigen, and rat anti-mouse CD140a fibroblast marker were both examined as potential sensitising antibodies but evoked no C3 deposition or lysis (data not shown).

Further studies using a calcein release assay allowed a more effective analysis of C-susceptibility in clonal cell lines. Cell death in this assay was again limited by the strong resistance to C-mediated killing. However, 10-15% of cells were lysed using this method. Cells were sensitised and treated with high doses of C *in situ* with dye release measured. Lysis of remaining cells

was achieved by treatment with detergent and used to generate killing curves based on percentage lysis. Despite cells remaining adherent throughout the experiment, a relatively high background lysis was observed in the absence of C. Background lysis values were subtracted from other dilutions to give an accurate estimation of C-mediated killing. The observed result that $C3^{-/-}$ cell lines appeared less susceptible to C-mediated lysis is likely due to the expression of CD59 conferring ability to regulate the terminal pathway and block assembly of the MAC. This is evidenced by the lower Kmax value observed for these populations. Values for $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ populations were also lower than WT, but were not as effectively protected as $C3^{-/-}$ lines. This experiment is also influenced by differing levels of sensitising Ab bound by these cells.

As described in Chapter 1, the abilities of CReg proteins to inactivate C3b and dissociate C3 convertases are highly significant for protection against C. Levels of C3b deposition can therefore provide a useful indication of C attack with increased deposition likely to correlate with increased C activation and/or reduced resistance capabilities. Detection of deposited C3b/iC3b was noted on all clones in a dose dependent manner, with maximal C3 deposited following incubation with 50% serum in all cases. The high MFI values at such concentrations indicated significant amounts of C3b/iC3b deposited but, as previously shown, these cells were not killed meaning that the cells remain intact even with large amounts of C3 products deposited on their surfaces. This observation is important to the hypothesis that tumour cells may in fact be stimulated by C deposition and exhibit enhanced proliferation or progression following a sublytic attack. Numerous cellular events are associated with both C3b and MAC deposition and are described fully in section 1.12. Thus the deposition of C fragments throughout these experiments may actually strengthen tumour cells, rather than just failing to cause lysis.

It is important to note that the rat anti-mouse C3 Ab used in this assay reacts with C3, C3b and also iC3b (including the breakdown fragment C3dg). The native C3 protein cannot bind to cells and requires cleavage through initial

activation of the AP, MBL pathway or, as in this assay, CP. Following initial C3b deposition, both the classical and alternative C3 convertases are involved with further deposition and amplification of the C response. iC3b is an 'inactive' product of factor I (fI) mediated cleavage and cannot contribute to continuation of the C pathway. Despite inactivity in terms of C-activation, iC3b remains an important molecule in terms of its role as an opsonin *in vivo* with the ability to contribute to immune recognition and clearance. Factor I is a serum protein and is a co-factor to numerous CReg (as described in Chapter 1). fI has been found to be secreted by fibroblasts (Vyse et al. 1996) and may be contributing to cleavage of C3 and protection from CDC in this model. Deposition of C3b and cleavage into inactive fragments is likely to be a key mechanism contributing to effects observed in Chapters 4 and 5 whereby C appeared to drive tumour proliferation. Persistent low-level C activation and C3b deposition may stimulate metabolic pathways which trigger proliferation and boost tumour growth. Rapid cleavage to inactive C3b breakdown products would protect cells from C-mediated lysis.

As evidenced by the data shown in Figure 6.22, clones of different genotypes were sensitised to different extents by the pAb. Relatively little variation was observed within each group with clones of the same genotype shown to bind similar levels of Ab. The difference was found to be statistically significant in each case. It is interesting to note that WT clones bind less Ab than each of the other groups since tumours were more readily induced in WT mice as described in Chapter 5. A possible explanation for this could be provided by enhanced classical pathway activation in WT tumours, stimulated via increased binding of Ab. However, flow cytometry and immunohistochemistry data failed to support this hypothesis although further investigation into the precise fragments deposited on different tumours is needed to fully quantify C activation and inactivation of deposited fragments by these tumours

Significant levels of C3b/iC3b were detected on each line assessed via flow cytometry. However, as described above, few cells are consequently lysed through this C activation. It seems likely that inactivation of C3 is a critical C regulatory protective mechanism of these cells as only CD59 is able to

regulate the terminal pathway with the majority of cells shown not to express this protein but remain highly resistant to CDC. Analysis of C5a could be used to determine initiation of the terminal pathway in this assay. A fair assessment of susceptibility to C attack on cell surfaces requires 'normalised' levels of sensitising Ab. As such, cells were treated with a range of sensitising Ab dilutions. Populations exhibiting equivalent levels of bound pAb were selected and incubated with serum. Calculation of EC50 values for clones allowed accurate comparison of susceptibility to C3b/iC3b deposition in this model. The data demonstrate statistically significant differences between groups, not evident from non-normalised data. The finding that WT clones required higher titres of serum to elicit equivalent levels of C3b/iC3b deposition is unexpected and does not easily fit with previous data showing the same populations to be marginally more susceptible to C-mediated killing. $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ clones were least susceptible to C3b deposition. The observed differences in C3b levels could be explained by differing proportions of C3b and iC3b, both bound by the Ab used in this assay. WT cells may have less total C3b/iC3b deposited than other clonal cell types though if a greater proportion was C3b (as opposed to inactive form), this could still contribute to enhanced killing. Further study of C3 fragments present on cell surfaces would be needed to investigate these hypotheses.

Although exhibiting effective resistance to C, and strong growth *in vitro*, these clones were unable to survive *in vivo* and were effectively cleared by both WT and $C3^{-/-}$ host immune systems. Due to the favourable pro-tumour environment which existed in the original tumour, these clonal lines may be poorly adapted to grow in a naïve mouse i.e. clones may be 'lethally immunogenic' and be cleared by immune responses. As discussed in Chapter 1, inflammation, cytokines and angiogenesis are key to the tumour micro-environment and contribute to tumour progression. These contributory factors help create a suitable environment to drive tumour survival and proliferation. In the absence of these factors, cultured and cloned lines may not survive. However, in order to rule out environmental factors, K6 was inoculated simultaneously into mice and found to exhibit different growth patterns to those observed in Chapter 4. Observed incidence was poor and inconsistent

under the same experimental conditions, endotoxin and mycoplasma contamination was ruled out suggesting that physiological changes in host mice were causing unreliable data. K6 tumour take was drastically reduced from ~90% (January 2006- April 2008) to ~40% (June 2008-November 2008) with tumours also arising later and exhibiting abnormally slow growth rates. However, $Rag^{-/-}$ mice which possess a severely compromised immune system (lacking in T-lymphocytes and B-lymphocytes) were also inoculated and showed regular and consistent K6 growth patterns. These data indicate a physiological alteration in immune responses to these cells by WT and $C3^{-/-}$ mice leading to enhanced clearance of fibrosarcomas. The anomalous findings coincided with the commencement of building works within the animal housing facility and increased noise and disruption to animals. Increased incidence of unexplained deaths within non-experimental mouse populations was noted and decreased litter sizes within breeding colonies. Increased stress levels may be present within animal populations leading to heightened immune responses effected through the release of various stress hormones. Such effects have been shown for increased leukocyte trafficking (Dhabhar et al. 2000), NK-cell activity (Dhabhar et al. 2000) and cytotoxic T-cell activity (Schild et al. 1999) through actions of heat shock proteins. However, the role of stress in immune responses remains a controversial subject with numerous findings of lower immune responses (reviewed by (Stefanski 2001)). Repeated attempts to achieve reliable data during this period were unsuccessful while various titres of fibrosarcoma cells were inoculated without significant improvement. These data are not shown here but involved titration of tumour load between 10^4 and 10^6 cells inoculated per host. Increased tumour dose was not sufficient to restore tumour take.

Following the return of fibrosarcoma growth in experimental mice (K6 observed to grow with ~80% incidence), lines generated as described in this Chapter were used to investigate differences in WT and $C3^{-/-}$ tumour growth in C3 sufficient and deficient mice. Time constraints prevented a full investigation of tumour growth fully utilising the WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}$.$CD59^{-/-}$ cell lines generated in this Chapter. However, each of these experiments would provide important data and remain novel and important

tools with which to work further in this area. The previous lack of tumour growth, and time limitations meant that only an initial investigation of one experimental parameter was possible. Consequently, it was decided to investigate the growth of WT and $C3^{-/-}$ tumour populations in WT (C-sufficient) and $C3^{-/-}$ (C-deficient) hosts. Additionally, heterogeneous (parent) lines were chosen for this experiment due to the higher tumour incidence and more robust model provided by these cells over clonal lines. Use of the clonal lines described above would have been preferable, however, to ensure that interpretable data with high incidence rates were generated in this final experiment, parent lines were selected. Two WT and $C3^{-/-}$ lines were inoculated into WT and $C3^{-/-}$ male mice with high incidence recorded. From 48 mice inoculated, only 6 failed to develop a tumour. This incidence level is ~88% and in line with K6 data described in Chapter 4. Data were presented and analysed in terms of incidence and survival using the logrank statistical test. However, each parameter analysed included 2 groups with relatively low group sizes (n=12). A trend was observed suggesting retarded tumour incidence following inoculation of $C3^{-/-}$ cells into $C3^{-/-}$ hosts, however low group sizes prevent this from achieving statistical significance and further investigation of these data is needed to elucidate any differences in fibrosarcoma growth rates. Such parameters are difficult to evaluate in the absence of a very strong trend and would likely require group sizes considerably larger than used in these studies. However, such experiments would be difficult to manage in terms of age-matched controls. Despite small group sizes used, these data are difficult to reconcile with previous data described in Chapters 4, 5 and above. It was shown that C3b/iC3b deposition observed in this model was derived solely from activation of host C, and that no C3, C3b or iC3b was detectable in tissue culture supernatant incubated with these cells *in vitro*. The lack of C3 secretion by tumours (as a deliberate attempt to drive tumour progression) suggests a difference would not necessarily be expected between *in vivo* progression of WT and $C3^{-/-}$ lines. However, experiments in Chapter 5 described pro-tumour effects attributed to host C3 activation, thus, a difference would be expected when inoculating either WT or $C3^{-/-}$ lines into Wt and $C3^{-/-}$ hosts through lack of pro-tumour effects in absence of host C3. However, these data are in agreement with

those in Chapter 4 and, as discussed, may be attributed to fast fibrosarcoma growth rates masking potential differences. Alternatively, C-driven tumour progression may be more relevant in growth of a developing tumour (i.e. 3-MCA induction, <200 days) than in an established tumour (i.e. cultured lines, < 28 days). Significant further work, involving use of all WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ clonal lines generated, sizable experimental groups and characterisation of arising tumours is required to further understanding of the roles for C-components or CReg in this model, while the use of the anti-C5 mAb may also help elucidate mechanisms contributing to or opposing tumour growth.

Post excision immunohistochemical staining of these tumours suggested that fibrosarcomas generated in this model do not secrete their own C3, or that levels are below the detection threshold. No C3/C3b/iC3b was detected in $C3^{-/-}$ hosts despite the same tumour populations exhibiting positive staining in WT hosts. This indicated that tumour cells activate host C rather than secrete tumour-derived C3 in this model. Taken alone, these data are insufficient to conclude that WT populations do not secrete C3 *in vivo*. The use of heterogeneous lines is advantageous for this reason, in that the populations comprise numerous fibrosarcoma sub-populations and thus the likelihood of C3-secreting cells is greatly improved. The use of just two WT lines in this experiment represents a clear limitation to the study, however, this finding substantiates previous findings in Chapter 4 (no C3b/iC3b detected on K6 cells in $C3^{-/-}$ hosts). This, and the *in vitro* lack of C3 secretion and C3 mRNA described provide convincing evidence against C3 secretion in this model.

Data described in this Chapter illustrate *in vitro* characteristics of WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ fibrosarcomas generated through 3-MCA tumour induction. Clones from each genotype were selected which exhibited comparable growth rates as determined by Alamar Blue metabolism over a 10-day period. Few differences were observed between groups with no CD55 expression or mRNA detected. Expression and RNA transcription of CD59 was conserved from heterogeneous populations only in $C3^{-/-}$ clones. No C3 secretion or mRNA was detected in any populations assessed. All clones

analysed were resistant to C-mediated killing (via CP activation) in both a flow cytometric and plate-based fluorescence assay. Further analysis of C susceptibility was performed via measurement of C3b deposition. Differences in Ab binding were controlled using differing titres of Ab per cell line, in order to bind comparable amounts on each. WT cells were shown to be less prone to C3b deposition, evidenced by higher doses of C required to evoke similar levels of deposition. Inoculation of 3-MCA inducted fibrosarcomas revealed no differences in incidence or survival in WT or $C3^{-/-}$ cells inoculated into WT and $C3^{-/-}$ hosts. However, the lack of C3/C3b/iC3b detected in/on tumour cells in $C3^{-/-}$ hosts appears to rule out the possibility of tumour-derived C3 in this model.

Experiments described in this Chapter were designed to establish and characterise novel clonal lines generated via 3-MCA tumour induction. Clonal lines of WT genotype, and those deficient in C components and CReg were generated and characterised *in vitro*. The *in vivo* characterisation of these lines remains incomplete yet represent valuable tools with which to continue investigation into the role of C in tumour growth and progression. Differences in tumour phenotype observed in WT or C-deficient host mice will be significant while data presented in this thesis demonstrates the validity of tumour induction and inoculation in the various mouse colonies available at Cardiff University as a means to investigate the important but poorly understood relationship between tumour cells and the C system.

7. Discussion

7.1. General Discussion

Experiments described in this thesis were devised to investigate whether components of the C system contribute to or restrict tumour growth. The hypothesis that C may promote tumour survival and/or proliferation is a relatively new concept and little convincing evidence is available to support this idea. In this thesis, two distinct techniques for investigating the hypothesis were employed. Firstly, a WT fibrosarcoma line was inoculated in WT mice, and mice lacking C1q, C3 or two potent CRegs (CD55, CD59). Further, the same line was used to assess the effect of C5-neutralisation on fibrosarcoma growth via use of a mAb. These experiments were performed to investigate the effects of specific C deficiencies on the *in vivo* incidence and growth of an established and pre-characterised tumour line. In order to evaluate the impact of C on tumour development *in vivo*, a second technique was employed. This involved injection of a chemical carcinogen (3-MCA) in WT mice and mice lacking C1q, C3 and the two CRegs. Comparisons of tumour incidence and progression in these groups of animals provided important insights into the roles of these proteins in *de novo* tumour development. A crucial further objective to 3-MCA induction of tumours was the generation of novel tumour cell lines of the same genotype to the host mouse. Thus, tumour cell lines with and without the ability to synthesise specific C-components or CReg were generated from excised tumours. These were characterised *in vitro* and re-inoculated into WT and $C3^{-/-}$ hosts in order that the impact of systemic C and tumour-derived C could be evaluated independently.

Results detailed in this thesis can be summarised into three key findings of particular significance:

1) The C component C5 can promote tumour progression following inoculation of an established fibrosarcoma line *in vivo*.

2) Both C components C1q and C3 can promote development of *de novo* tumours following 3-MCA tumour induction *in vivo*.

3) $CD55^{-/-}.CD59^{-/-}$ hosts exhibit significantly impaired development of *de novo* tumours following 3-MCA tumour induction *in vivo*.

7.2. A pro-tumour role for the C5 protein

Experiments detailed in Chapter 4 describe the use of an established WT tumour cell line, K6. This fibrosarcoma line was injected into mice and tumour growth monitored. In the case of C1q and C3 deficiencies, no differences in tumour progression were observed with tumours growing rapidly and reproducibly in groups of WT, $C1q^{-/-}$ and $C3^{-/-}$ mice. However, *in vivo* neutralisation of the C component C5 revealed a pro-tumour effect associated with this protein. In the absence of C5, hosts exhibited a statistically significant retardation in tumour growth compared to mice treated with an isotype control antibody. This effect, together with the observed deposition of C3 activation fragments on tumours in both WT and C5-neutralised mice indicated that one or more C pathways were activated in the inoculated animals and that C5 cleavage or downstream events were responsible for the pro-tumour effect in WT animals. Release of the anaphylatoxin, C5a, or deposition of the MAC, seem the likely candidates to enhance tumour progression in WT mice, each of which is absent in C5-neutralised counterparts. Possible mechanisms responsible for the pro-tumour effects are described below.

Although work in this area is at an early stage, a very recent study observed a similar pro-tumour role for C5 cleavage mediated through the pro-inflammatory effects of the C5a anaphylatoxin (Markiewski et al. 2008). In this model, a murine cervival carcinoma cell line (TC-1) was used to identify a role for C in tumour progression. Elucidation of the contributory mechanism was achieved by use of an antagonist for the C5a receptor (C5aR) and separately by the use of $C5aR^{-/-}$ mice. In each case, the pro-tumour effects were abrogated in the absence of C5aR signalling and tumour growth retarded. Further, the role of C5a and C5aR interactions in the migration and immunosuppressive activity of myeloid derived suppressor cells (MDSCs) was demonstrated. These cells were more abundant in tumours derived from WT animals compared with those where the interaction between C5a and C5aR was blocked. Accumulated MDSCs were shown to suppress the immune response in WT hosts by dampening CD8+ anti-tumour responses thereby contributing to enhanced tumour growth. Therefore, the roles of C5a as a chemoattractant for MDSCs and in the promotion of inflammation were implicated in driving tumour progression in this model. Importantly, Markiewski et al also reported C1q deposition on tumour surfaces and impaired tumour growth in $C3^{-/-}$ and $C4^{-/-}$ animals thus implicating classical pathway activation in the generation of C5a and pro-tumour effects.

As described above, a pro-tumour role for C5 was also shown in the experiments described in Chapter 4. These data initially appear to support the findings by Markiewski et al and could also be attributable to a lack of C5a signalling, recruitment of MDSCs and impaired $CD8^{+}$ T-cell responses in C5-neutralised mice. However, key differences are apparent between the observed results and a separate mechanism appears to be responsible for the effects in this study. Results described in this thesis appear independent of classical pathway activation as deficiency of C1q did not impair tumour growth. Although activation of any C pathway allows generation of C5a, this observation suggests a different mechanism of C activation by tumour cells in this thesis. Further, the comparable tumour progression observed in WT and $C3^{-/-}$ mice suggests a protective role for C5-neutralisation independent of C since no evidence for C5-cleavage was provided in $C3^{-/-}$ mice. If C-activation

was crucial to the observed effects, a similar protective effect would be expected in $C3^{-/-}$ mice. Data shown in 7.1 represents key similarities and differences between a recent study by Markiewski et al, and results obtained in this thesis. Both reports describe a hitherto un-suspected role for the C5 protein in progression of tumours *in vivo* but appear to be via different mechanisms. However, a major difference between the two models is the cell line utilised to assess tumour growth. TC-1 is a cervical carcinoma line whereas K6 is a fibrosarcoma line. These lines may utilise different mechanisms to avoid immune clearance and may also stimulate C-activation via a different pathway. Generation of the C5a fragment is known to exhibit chemotactic effects on fibroblasts (Postlethwaite, Snyderman and Kang 1979), and also on several other tumour cell types (Orr et al. 1983). This effect was shown to be C5a specific and no chemotaxis was observed following C3a release. This mechanism has been also implicated in enhanced adhesion and metastasis in a rat carcinosarcoma model (Orr, Mokashi and Delikatny 1982).The above reports represent a possible C-independent mechanism for enhanced tumour growth following C5a release and represent a potential mechanism independent of C3a generation. However, C5a generated through C activation pathways also necessitates C3a release, making it difficult to elucidate effects specific to C5a in this thesis. Data described in Chapter 4 appear consistent with the above reports and suggested a pro-tumour role for C5a release.

Study	Host	C3 cleavage	C5a effects	MAC deposition	Effect on tumour growth
Markiewski et al (2008)	WT	✓	✓	✓	normal
	$C3^{-/-}$	x	x	x	impaired
	$C5aR^{-/-}$	✓	x	✓	impaired
This thesis	WT	✓	✓	✓	normal
	$C3^{-/-}$	x	x	x	normal
	$C5_{neut}$	✓	x	x	impaired

Table 7.1: Summarised similarities and differences in tumour progression following inoculation of tumour cell populations into WT mice or those lacking C-components C3 or C5.

Whilst data presented in Chapter 4 indicate a pro-tumour role for C5 independent of C3, the use of $C3^{-/-}$ knock-out mice and C5-depleted hosts may also be of significance. This is due to differences in histocompatibility, cells derived from a C57Bl6 mouse (K6) will be genetically identical to those on the same background (i.e. WT). However, $C1q^{-/-}$ and $C3^{-/-}$ knock-out mice were originally generated on different backgrounds and backcrossed onto the C57Bl6 background. Although every care was taken to minimise histocompatibility issues (by backcrossing a minimum of 10 generations) – subtle differences may remain significant and cause an immune response and tumour rejection. The use of a C3-depleting agent or other convertase-inhibiting therapies in the current model would allow comparison of littermate controls, eliminating histocompatibility issues and providing further control and support for the above findings.

7.3. 3-MCA tumour induction

Experiments described in Chapter 5 involved the use of a chemical carcinogen (3-MCA) to induce tumours in male mice. Tumours grew in 100% of WT mice injected, but growth was inhibited in $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}$.$CD59^{-/-}$ mice suggesting important roles for these proteins in promoting tumour progression *in vivo*.

7.3.1. Impaired 3-MCA tumour induction in $C1q^{-/-}$ hosts

For the first time, experiments performed in Chapter 5 revealed pro-tumour roles for the initiator of classical pathway activation C1q and the central C-component C3 following injection of a carcinogen. As described, $C1q^{-/-}$ mice formed tumours later and with lower incidence than WT controls. Evidence presented throughout this thesis suggests that fibrosarcoma cells activate C *in vivo* suggesting that tumours raised in $C1q^{-/-}$hosts, unable to activate the classical pathway, may activate C to a lesser extent and experience lower levels of the cellular activation events associated with C3b/MAC deposition and described in section 1.12. However, this hypothesis was not supported by data in Chapter 5 whereby comparable levels of C3b/iC3b deposition were

observed on WT and C1q$^{-/-}$ tumour tissues. Deposition of C3 activation fragments on tumour sections cannot be derived from classical pathway activation in C1q$^{-/-}$ hosts and either alternative or MBL pathways must be activated. Whilst C3 activation products may be deposited via the same pathway in WT and C1q$^{-/-}$ tumours, it is also possible that deposition on WT tumours is derived from the classical pathway and derived from alternative pathway activation on C1q$^{-/-}$ tumours through a different mechanism. Deficiency of C1q is linked to impaired clearance of apoptotic cells and consequent activation of the alternative pathway (Matsui et al. 1994, Mevorach et al. 1998). In order to determine the mechanism of C activation in C1q$^{-/-}$ tissues (i.e. tumour activated or apoptotic cell activated), assessment of apoptotic cell numbers in C1q$^{-/-}$ and WT cell populations is needed and may be achieved via immunohistochemical techniques. Commonly used markers for labelling apoptotic cells include caspase 3, a cysteine proteinase enzyme central to the apoptotic pathway. Alternatively, an established method exists for detection of DNA fragmentation known as terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (Gavrieli et al. 1992). This technique may be adapted to accurately identify cells in the latter stages of apoptosis. Co-localisation of C3b deposition with apoptotic cells would suggest these to be responsible for C-activation while decreased infiltration of phagocytic cells may also be observed in C1q$^{-/-}$ mice compared to WT controls. Elucidation of the mechanisms of C activation by tumours or apoptotic cells is a critical further experiment and is necessary in order to better interpret the role of C1q in tumour progression.

A role for C1q in controlling tumour growth has also been shown independent of CDC. A murine tumour study previously found that local C1q biosynthesis was required for both Ab-mediated and Ab-independent tumour cytotoxicity through activation of macrophages (Leu et al. 1990). Additionally, a recent report that C1q can induce apoptosis in human prostate cancer cells also argues against a pro-tumour role for C1q (Hong et al. 2009). These reports of anti-tumour roles for C1q are in contrast to data presented in this thesis and predict enhanced tumour growth in C1q$^{-/-}$ mice. These effects are either

irrelevant in the 3-MCA tumour induction model, or are outweighed by factors promoting tumour growth in WT mice.

7.3.2. Impaired 3-MCA tumour induction in $C3^{-/-}$ hosts

A role for the central component C3 was also demonstrated in facilitation of tumourigenesis following 3-MCA injection. This finding appears to support observations in $C1q^{-/-}$ mice and may also be linked to impaired classical pathway activation in $C3^{-/-}$ mice and suggests a pro-tumour role for the C3 protein. This observation is particularly important given the diverse roles for the C3 protein in both innate and adaptive immunity, in particular the contributions to optimal T-cell and B-cell responses (Kerekes et al. 1998), (Carter and Fearon 1992). These mechanisms are discussed fully in section 1. 4). Given these roles, any pro-tumour role for C3 would be expected to be offset or eclipsed by significantly impaired immunosurveillance in $C3^{-/-}$ mice resulting in enhanced tumour progression compared to WT controls. However, this was not the case and the tumour progression was restricted in $C3^{-/-}$ hosts. Thus, impairment of immune responses associated with lack of the C3 molecule either did not affect tumour growth, or the effects were significantly outweighed by the absence of pro-tumour effects of C3 present in WT hosts. Clear evidence demonstrating the importance of C in tumour progression is provided and necessitates a change in conventional thinking and significant further study in this area. As yet, there is scant other evidence to support possible mechanisms for a pro-tumour role for C3, however, a study on mouse lung carcinoma cells demonstrated enhanced proliferation following C3 activation, surface deposition and internalisation thought to be mediated via a stimulatory growth signal (di Renzo et al. 1999). Further work involving the use of anti-C3 Abs proved a direct role for C3 in enhanced tumour growth. Internalisation of C3 products was followed by increased protein kinase activity with the PKC family crucial to proliferative effects observed (Longo et al. 2005). Activation of cellular pathways and increased phosphorylation by various tyrosine kinases may lead to a multitude of downstream effects capable of stimulating cells and promoting tumour growth. The reports detailed in section 1.12 whereby C3b and MAC deposition may

activate these pathways represent a possible explanation for the enhanced tumour progression observed in WT compared to $C3^{-/-}$ hosts.

Whilst both C components C1q and C3 were shown to be important for driving tumour progression following induction by 3-MCA, pro-tumour effects for these proteins were not observed in Chapter 4 following inoculation of cultured fibrosarcoma cells. In these experiments, C5 only was shown to contribute to enhanced tumour progression with comparable growth in WT, $C1q^{-/-}$ and $C3^{-/-}$ hosts. As previously discussed in Chapter 4, the limited timescale of these experiments (<4 weeks as opposed to <4 months in 3-MCA experiments) may be insufficient for pro-tumour effects to become apparent. Alternatively, differences between the two tumour growth models may be responsible and suggesting a role for C in promotion of developing tumours, but not in established populations i.e. the early events related to tumourigenesis may be aided by C activation whereas developed tumour cells may grow sufficiently well in the presence or absence of C. In order to investigate this hypothesis, alternative models could be employed utilising different cell types or tumour induction models i.e. varying the tumour incubation time by using a less aggressive cell line. For example, the murine melanoma cell line B16 is also derived from a C57/BL6 mouse (thus avoiding antigen differences) and exhibits a longer latency period that K6 with slower growth and hosts remaining tumour free up to 3 weeks post inoculation with 10^5 cells (Cuff et al. 2010).

7.3.3. C as a 'double-edged sword' in tumour immunity

An important consideration when evaluating C-related effects is that depletion or deficiency of a protein not only abrogates the effects associated with (deposition or cleavage of) that protein, but also affects all downstream activation events. In this way, $C3^{-/-}$ mice show no C3b deposition on tumour cells, but tumour cells will also not activate the terminal pathway. Therefore C5a release and MAC deposition are also absent in $C3^{-/-}$ hosts, and attributing a pro-tumour role to a single C-component is difficult. Cellular activation events following C3b / MAC deposition represent a possible mechanism for

pro-tumour effects associated with C. These effects are discussed in section 1.12 and represent ways by which nucleated cells exhibit increased protein kinase activity following sublytic C deposition and subsequent recovery of targeted cells. Further, cells exposed to sublytic doses of C may acquire increased resistance to subsequent C deposition, known as C-induced protection. The concept of induced protection was not studied in this thesis and it's role remains unclear *in vivo*. However, this phenomenon represents a significant mechanism which may contribute to a pro-tumour role for C and warrants renewed investigation. Ideally, tumour cells would be treated with different levels of C deposition *in vivo* with proliferative rates, CReg expression and tendency to metastasise assessed. This may be achieved via the use of differing Ab doses to activate classical pathway although considerable care must be given to control for non-C effects i.e. ADCC. Further *in vitro* work is also needed to further elucidate effects and mechanisms associated with sublytic C attack. Considerable work in this area allowed determination of numerous cell effects (discussed in Section 1.12) associated with sublytic C deposition and recovery, however, further study in this area to investigate specific effects and also explore whether tumour cells can exploit these mechanisms or they actually exhibit equivalent outcomes to non-transformed nucleated cells. In order to study the effect of *in vitro* sublytic attack, cells may be treated with C prior to inoculation into WT hosts, with differing doses of C tested and *in vivo* growth of tumour cells monitored. Fibrosarcoma cells could be used to investigate this hypothesis, although data described in Chapter 6 show these cells to be highly resistant to CDC. Consequently, a cell line sensitive to C *in vivo* would be extremely interesting to assess whether initial treatment could render the inoculated cells more resistant and aid progression. Further generation of fibrosarcoma cells as described in this thesis may produce such a cell line, whilst other tumour cell types may also be more suitable. These studies could then be extended to test for effects of specific components as detailed in this thesis, this would include deposition of C3b fragments in the absence of MAC e.g. C5-depleted.

An inherent problem with the use of C-deficient animals is the inability to attribute observed effects to a single mechanism. This is due to the cascade

nature of the C system which means that deficiency in a certain C-component may yield effects attributable to that protein or to any of the numerous downstream effects. Thus, both pro-tumour and anti-tumour effects of C will be affected by deficiency in any C component. In reality, an overall pro-tumour effect of C1q and C3 deficiency is shown in Chapter 5 but this effect is comprised of a number of variables and an overall representation of the 'balance' between pro-tumour and anti-tumour responses is apparent. Figure 7.2 outlines several of the major contributory factors involved with C1q and C3 deficiency and in each case, the pro-tumour role for C (in WT hosts) outweighs the various immune mechanisms contributing to anti-tumour responses. Isolation of clearly defined roles for individual proteins (e.g. C5a) is necessary to properly address this emerging hypothesis and evaluate possible therapeutic strategies to counteract pro-tumour effects associated with C.

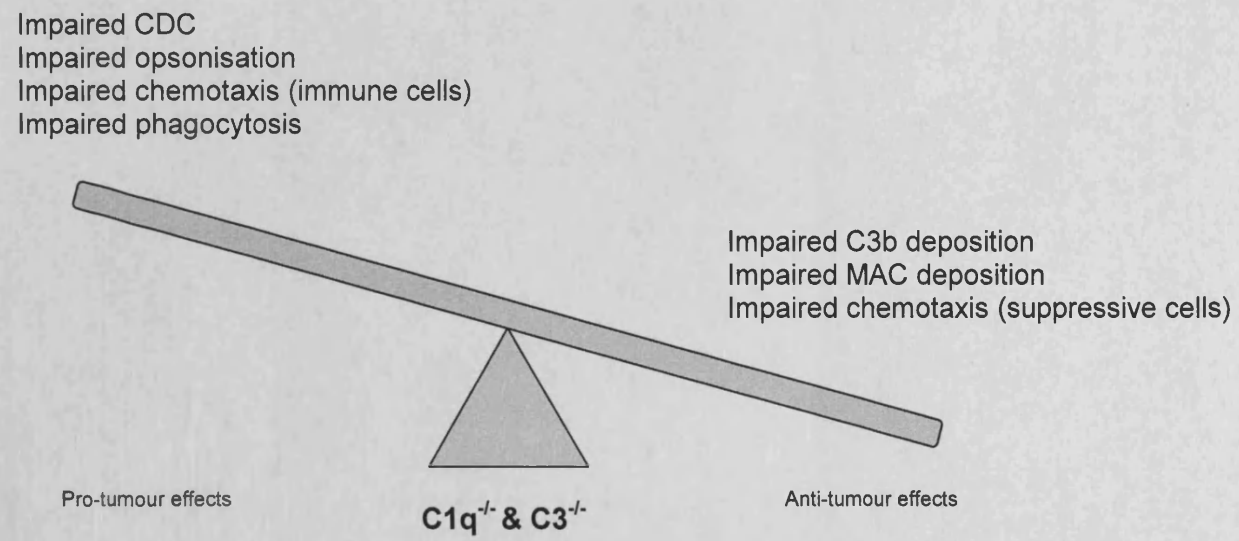


Figure 7.2: Schematic diagram representing 'balance' of pro- and anti-tumour effects associated with deficiency of C1q or C3 in 3-MCA tumour induction.

The inflammatory response is an important factor in tumour development and has long been thought to contribute to cancer progression. Several studies have found a decreased cancer risk in human patients prescribed anti-inflammatory drugs over a long period of time (García-Rodríguez and Huerta-Alvarez 2001, Meier, Schmitz and Jick 2002). As described previously, release of anaphylotoxic protein fragments C3a and C5a are consequences of C activation with potent pro-inflammatory effects attributed to both. In this way, activation of C may contribute to pro-tumour effects through increased inflammation not present in C5-depleted hosts. The protective effects observed associated with C1q and C3 deficiency were strong and statistically significant yet appear to be mediated through separate mechanisms. Whilst the work in this thesis aims to investigate pro and anti-tumour effects of C, the observed results point to a strong role for C in tumour progression despite impairment of various immune mechanisms. The classical pathway of C is known to be important for induction of optimal immune responses. C1q has been shown to be important in optimal T-cell responses with defective cytokine production noted in $C1q^{-/-}$ mice (Cutler et al. 1998). Additionally C3 is strongly implicated in optimal T-cell responses (see Section 1.4) (Arvieux, Yssel and Colomb 1988, Kerekes et al. 1998). However, both $C1q^{-/-}$ and $C3^{-/-}$ mice exhibited retarded tumour growth even with impaired immunosurveillance mechanisms. This indicated strong pro-tumour roles for these proteins able to overcome impairment to anti-tumour responses and develop statistically significant effects against tumour progression.

Deposition of C3b/iC3b and MAC was observed on tumour samples grown in WT, $C1q^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ mice. The tissue analysed from these mice was from progressively growing tumours and therefore those not cleared by immune mechanisms. Preferably, host mice in which tumours grew initially, but were cleared by C-responses would also be analysed. These C-sensitive cells would be isolated and characterised (e.g. CReg expression, MAC shedding, C3 secretion) to identify differences in cleared and non-cleared tumour cell populations. Thus, mechanisms by which tumour populations were able to escape immunosurveillance could be identified. Unfortunately, such evaluation is inherently difficult as palpable tumour populations rarely

arise in these animals and cannot be excised and evaluated. Importantly, tumours in $C3^{-/-}$ hosts were not subjected to C3b or MAC deposition, nor could C3a or C5a be generated. A role for C5a-mediated recruitment of MDSCs (in WT hosts) is therefore possible in this model as described above and demonstrated by Markiewski et al (2008). Markiewski's study showed retarded tumour progression in $C3^{-/-}$ mice through impaired recruitment of MDSCs in the absence of C5a. Unfortunately, it was not possible to investigate the effects of C5 deficiency/depletion using 3-MCA to induce tumours in this study. This was due to the large quantities of mAb required to fully deplete host mice, and the extended time period (>90 days) before tumours arise in this model. For this reason, knock-out animals rather than antagonists or depleting antibodies are more appropriate to examine effects on 3-MCA tumour induction. However, depletion of C components could be achieved over a shorter time span e.g. to examine the effects of C5 during a 2 week window immediately following injection of the carcinogen. This could allow assessment of the role of C5 in the early events associated with carcinogenesis. Investigation of C5-deficiency in the 3-MCA model would be an important experiment to consider and examine the role of C5a in inflammation during tumour development and immunosurveillance. A pro-tumour effect would be expected to support findings in Chapter 4 and may help elucidate a mechanism for the effects of C3-deficiency observed in Chapter 5.

7.3.4. CD55 and CD59 in tumour progression

Also investigated in Chapter 5 was the effect of CReg deficiency on 3-MCA induced tumours. Mice deficient for both CD55 and CD59 were injected with 3-MCA and found to be resistant to tumour induction. Various tumour cells are thought to abundantly express CReg in order to protect themselves from C-mediated lysis (Fishelson et al. 2003), thus, the inability to express such proteins may be expected to sensitise emerging tumour cells to CDC. However, characterisation of tumour populations revealed that CD55 was not expressed on WT, $C1q^{-/-}$ or $C3^{-/-}$ tumour cells. This observation was made immediately post excision of tumour cells with the lack of CD55 expression

also maintained following generation of *in vitro* populations and clonal cell lines. Therefore, CD55 initially appears not to contribute to tumour progression in this model. Conversely, the expression of CD59 was demonstrated in WT, $C1q^{-/-}$ and $C3^{-/-}$ groups immediately following tumour excision. This suggests that tumour expressed CD59 is more important in this model and could be responsible for the preferential tumour growth observed in WT compared to $CD55^{-/-}.CD59^{-/-}$ hosts. In these mice, tumour cells did not materialise or were cleared effectively and the lack of CD59 expression is likely to sensitise fibrosarcomas to CDC through inability to regulate the terminal C pathway. However, alternative roles for these CReg other than protection from CDC have recently been demonstrated which may are important when considering the observed results. Although CD55 was not present on excised tumour cells from WT animals, expression on other (non-tumour) cells may instead be significant. Deficiency of CD55 has been shown to allow stronger CD4+ and CD8+ T-cell responses to antigen (Liu et al. 2005, Fang et al 2007), which could lead to enhanced clearance of tumour cells in $CD55^{-/-}$ mice. A similar down-modulatory role for CD59 has been shown in suppression of CD4+ T-cell responses leading to decreased proliferation of these cells (Longhi et al. 2005). Again, $CD59^{-/-}$ mice may therefore exhibit enhanced clearance of tumour cells and protection from 3-MCA tumour induction. The global lack of these two proteins could therefore lead to enhanced anti-tumour immune responses and tumour clearance dependent on C activation, but not via CDC. These novel roles for CReg are an area worthy of significant further study and are yet to be investigated in the context of tumour immunology. The respective effects which may contribute to the lower tumour incidence in $CD55^{-/-}.CD59^{-/-}$ mice are summarised in Figure 7.3. Significant further work would be required to investigate this hypothesis with differing contributions of CD4+, CD8+, NK-cells expected depending on the tumour model used. Infiltration of these cells could be assessed by immunohistochemistry or flow cytometry, however, careful consideration must be given to the activities of these cells (e.g. large numbers of cells may suggest a response, but activity may be suppressed and cell presence irrelevant). Activation markers could help identify anti-tumour responses, while isolation and *in vitro* characterisation for cytokine production and/or responses

to tumour antigen would also be important. The precise immunological functions of CD55 are yet to be established with a role for this protein also reported in enhancement of T-cell responses. Cross-linking of CD55 using mAbs has been shown to activate T-cells (Tosello et al. 1998). It has been suggested that the enhanced T-cell responses are caused by abrogation of CD55 CReg function, leading to increased production of C activation fragments. This is consistent with the findings of Liu (2005) and Heeger (2005) who noted enhanced T-cell responses in $CD55^{-/-}$ mice. However, a further demonstration of CD55-mediated enhancement of T-cell responses (in conjunction with TCR engagement) found increased activation of CD4+ T-cells via a non-neutralising mAb specific for CD55 suggesting a role independent of C deposition and diverse roles for the CD55 protein on T-cells (Capasso et al. 2006)

Data described in this thesis suggested possible roles for CD55 or CD59 in promoting tumour progression, the reasons underlying this difference must be determined. If enhanced T-cell responses are responsible for tumour clearance in $CD55^{-/-}.CD59^{-/-}$ hosts, this could represent a crucial strategy in harnessing existing host immune mechanisms to clear tumour cells. Initial investigation of CD4+ infiltrates within tumours did not identify a difference between groups though investigation of T-cell phenotype (T_{helper}, T_{reg}, CD8+) and anti-tumour activity of these cells would provide crucial data as to the immune effectors contributing to the observations. Further, the use of single knock-outs (i.e. $CD55^{-/-}$ and $CD59^{-/-}$ separately) is important to examine the effects of individual CReg deficiencies and allow dissection of the relative contributions of CD55 and/or CD59 to pro-tumour effects in WT mice. Whilst various approaches for targeting CReg on tumour surfaces are available and in development (Ziller et al. 2005, Macor et al. 2007) perhaps a more successful strategy may include targeting CReg expressed on immune cells to temporarily increase their anti-tumour functions. When combined with traditional tumour therapy e.g. excision, radiotherapy and chemotherapy treatments, enhancement of the efficacy of circulating immune cells may help prevent relapse or metastasis and improve patient prognosis. However, such an approach is likely to have significant autoimmune consequences and

would require extensive research to assess, optimise and control potential effects.

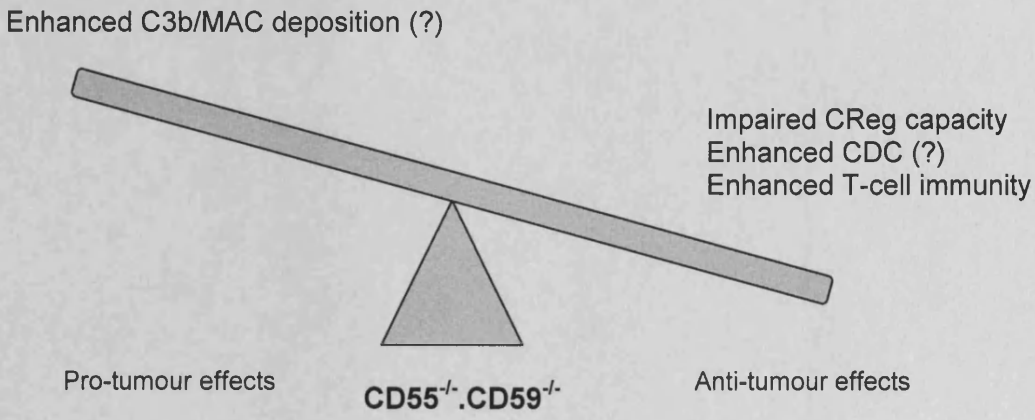


Figure 7.3: Schematic diagram representing 'balance' of pro- and anti-tumour effects associated with deficiency of CD55 and CD59 in 3-MCA tumour induction.

7.4 Further observations

Further to the three key findings described in this thesis, a number of other observations provided useful insights into the role of C in tumour progression. Experiments detailed in Chapter 6 further confirmed the observation from previous Chapters that fibrosarcoma cells do not express the CReg CD55. This absence is somewhat surprising but may be linked to the abundant expression of Crry detected on all tumour cells analysed, which has an overlapping CReg function with CD55. While all tumour populations expressed CD59 *in vivo*, only $C3^{-/-}$ lines were shown to conserve this expression *in vitro.* Preliminary experiments were performed in order to identify differential growth of these cell lines in C-sufficient, and C-deficient hosts. Re-inoculation of WT and $C3^{-/-}$ lines failed to reveal a difference in tumour progression in either WT or $C3^{-/-}$ hosts, although increased group sizes would be required in order to confirm this observation. Unforeseen problems associated with tumour growth in this model and time constraints prevented a full investigation of host and tumour derived C components and their *in vivo* effects. These experiments remain incomplete and represent an invaluable opportunity to assess the effect of tumour cell C deficiencies on

tumour growth. Further, assessment of CReg expression following re-inoculation may reveal that WT cells up-regulate CD59 to previous levels when returned to an *in vivo* setting. Clonal fibrosarcoma cell lines (WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$) were generated and extensive study of the progression of these cells in WT and C-deficient hosts are important experiments with the potential to elucidate key mechanisms by which C can promote tumour progression *in vivo*. The secretion of C-components e.g. C1q and C3 by tumour cells may play important roles in tumour progression and represent a clear strategy by tumour cells to induce cellular activation mechanisms. Cell lines sufficient and deficient in proteins are important tools to address the contributions of host and tumour derived C component secretion to tumour progression. The re-inoculation of $CD55^{-/-}.CD59^{-/-}$ cells also represents an important further experiment with interesting consequences. These cells would ordinarily be expected to be sensitised to CDC through impaired regulation of activation pathways (CD55) and the terminal pathway (CD59). However, a role for tumour expressed CD55 was not shown for these fibrosarcoma lines, and CD59 only was detected on WT tumour cells. Cells lacking these CReg may be poorly adapted to *in vivo* growth, cleared by CDC and account for the poor tumour growth in $CD55^{-/-}.CD59^{-/-}$ described in Chapter 5. However, in light of more recent immunological roles shown for CD55 and CD59 (reviewed by Longhi et al. 2006), these effects may be independent of CDC. Therefore, these cells may up-regulate different CRegs, or utilise alternative mechanisms to avoid lysis upon re-inoculation into C sufficient animals thus remaining protected from C effects and able to proliferate rapidly. Responses of these cells to C deposition would provide crucial insights into the value of C as a potential anti-tumour effector mechanism.

7.5. C3 secretion

The secretion of C components by fibroblast cells (Garred et al. 1990) , and several tumour cell types (Kitano and Kitamura 1993, Jean et al. 1997) has previously been demonstrated. The central component C3 is the most commonly found C-component in culture supernatants and may be an important factor in the relationship between tumour cells and C. In order to address the relative contributions of host and tumour derived C components to tumour progression, cells which synthesise and secrete C components are required. Synthesis or secretion of C3 by WT cells generated by 3-MCA tumour induction was not observed in this study, but may require stimulation before C3 can be detected. This may be achieved *in vitro* via treatment with various stimulatory cytokines e.g. IL1, IL6 and IL13 (Katz and Strunk 1989, Katz, Revel and Strunk 1989, Katz et al. 1995). However, the absence of C3b/iC3b deposition on WT tumour cells in $C3^{-/-}$ hosts as described in Chapter 6 suggests that these cells were not stimulated to secrete significant C3 *in vivo* with no C3b/iC3b detected on tumour sections following re-inoculation. This may be due to very low amounts of C3 being secreted, or that C3 was not activated and was unable to deposit on cells. Generation of tumour cell lines which secrete C3 (and those generated by identical methods in $C3^{-/-}$ hosts) would provide an invaluable tool with which to investigate the role of tumour derived C and enhanced proliferative or metastatic effects associated with such a phenotype. A number of C3 cleaving proteins have been identified which are secreted by tumour cells, these cysteine proteinase enzymes have C3 inactivating capacity and have been found to be co-secreted with tumour derived C3. This represents a possible mechanism by which tumour cells may promote their own progression, through C3b deposition and subsequent cellular activation, yet remain protected from CDC and opsonisation by enhanced C3 cleavage capacity. Up-regulated or enhanced C3 inactivation may be a feature of tumour cells secreting C3 and remains an area worthy of further study. While C3 secretion is the best studied and provides the most likely mediator of pro-tumour effects, investigation of various other C-component and CReg expression or secretion is worthwhile and may yield interesting results.

7.6. Further experiments

In addition to WT, $C1q^{-/-}$, $C3^{-/-}$ and $CD55^{-/-}.CD59^{-/-}$ animals and tumour populations generated from these hosts, a number of other knock-out mice would be extremely useful to further dissect the contributions of individual C components to tumour progression. The model employed in this thesis provided a powerful and reproducible method allowing comparison between WT and C-deficient animals. Differences observed became statistically significant with manageable group sizes and provided interesting observations. Of particular interest would be mice deficient in C2, C4 or fB, this would allow assessment of the relative contribution of the classical (C2 or C4) and alternative (fB) activation pathway to C activation, deposition and tumour progression. Whilst C1q remains the most significant C-component linked with apoptotic clearance, further investigation of apoptosis in the tumour micro-environment and effects on tumour growth could be achieved by study in $C2^{-/-}$ or $C4^{-/-}$ mice given the reported roles for classical pathway components in clearance of ACs (Taylor et al. 2000). Additionally, mice deficient in a terminal pathway component e.g. C6 would provide an excellent opportunity to assess the lytic pathway i.e. effects of MAC deposition. Crucially in this deficiency, release of the anaphylatoxin C5a is possible, but no further deposition of C components onto target membranes can occur. Mice deficient in fB and C6 are available in house, currently being back-crossed onto a C57Bl6 background and will provide exciting opportunities to extend this work when properly controlled with WT mice on the same background. Additionally, the respective contributions of the CReg, CD55 and CD59, to the protective effect can also be elucidated via experiments with single knock-outs. Such experiments would involve induction of tumours (via 3-MCA) in WT, $CD55^{-/-}$ and $CD59^{-/-}$ tumours. A statistically significant difference would be expected between WT and deficient groups depending on whether CD55 and/or CD59 was responsible for the pro-tumour effect in WT hosts observed in Chapter 5. At present, effects cannot be attributed to either protein with evidence to suggest both can contribute to enhanced tumour progression.

An exciting recent development offers an opportunity to extend this study in $Crry^{-/-}$ mice allowing investigation of both the potent C-regulatory (Section 1.6.2.4) and immunological roles (Section 1.7.3) of this crucial protein. Previously, mice bred to this genotype died *in utero* and could be studied only by cross-breeding to a $C3^{-/-}$ background. However, $Crry^{-/-}$ mice have recently been generated following inhibition of the terminal pathway (Ruseva et al. 2009), these animals are able to synthesise and deposit C3 activation fragments. Clearly, Crry is of vital importance in mice and was expressed on all tumour sections analysed in this study. A role for Crry expression has been shown in promotion of tumour metastasis in a rat model of colorectal carcinoma, with significantly decreased outgrowth following blockade of Crry (Gelderman et al. 2004). The Crry protein appears important in murine tumours, and fibrosarcomas deficient in this CReg would be invaluable to investigate resistance to CDC and Crry contribution to tumour progression.

Further roles in immune cell signalling are becoming apparent with promotion of differentiation and activation demonstrated for mouse CD4+ T-cells following co-ligation of CD3 and Crry (Fernández-Centeno et al. 2000, Jiménez-Periañez et al. 2005). Another important finding was provided by studies whereby transfection of rat adenocarcinoma cells with Crry enhanced tumourigenicity and decreased ADCC through impaired NK-cell responses (Caragine et al. 2002). Importantly, this function was shown to be independent of C-opsonisation and revealed a novel role for Crry in mediating NK-cell immunity. Therefore the role of Crry in tumour progression extends outside it's role in regulating complement, and $Crry^{-/-}$ mice will allow further investigation into a purported role in tumour progression in rodents and how that may translate into human studies.

Experiments described in this thesis were designed to investigate the effects of specific C components or CReg on tumour progression. Further, several key characteristics were identified and assessed including expression of the CRegs CD55, CD59 and Crry. However, various other CRegs exist which may contribute, or be crucial to tumour cell survival and/or growth and represent important future experiments to further the characterisation of these

cells and lead to better understanding of the relationship between fibrosarcoma cells and the C system. Expression of CR1 is normally restricted to B-cells and DCs in mice (Kinoshita et al. 1988), but retains C regulatory activity and could potentially be utilised by tumour cells, while fluid phase CReg such as fH, fI or C4bp could also serve to regulate classical and alternative pathway activation respectively. A number of cell surface markers may also provide useful indications of mechanisms affecting tumour growth in this model. For example, reduced MHC Class I expression has been implicated in escape from immunosurveillance through impaired antigen presentation and T-cell mediated immunity (reviewed by Bubenik J et al. 2004). Additionally, expression of C component receptors (e.g. C1qR, C3aR, C5aR) by both tumour cells, and infiltrating immune cells could be of significance in order to elucidate contributory pro-tumour or anti-tumour effects. Such characterisation was performed by Markiewski et al and was able to identify the recruitment of immunosuppressive cells (MDSCs) by chemotaxis through expression of C5aR.

As described in section 1.10, and as demonstrated in Chapters 4, 5 and 6, fibrosarcoma cells are able to activate the C system which is therefore considered a potential anti-tumour effector mechanism (reviewed by Macor et al. 2007). A major advantage of utilising C in Ab-targeted anti-tumour therapy involves the local biosynthesis of C components in the tumour vicinity and the enhanced penetration of small, soluble components into tumour masses compared with impaired penetration by immune cells, thus the localisation of C components to targeted tumour cells is easier than that of other ADCC effector cells e.g. NK-cells. Additionally, a crucial feature of the classical C pathway is the ability to localise activation to the site of Ab binding. As such, mAbs specific for tumour antigens may localise C deposition to tumour surfaces and cause tumour cell death by CDC or opsonisation. However, as previously described tumour cells possess numerous mechanisms to evade C-mediated cytotoxicity, the best studied being expression of CReg. As such, mAb immunotherapy remains an inefficient yet promising anti-tumour treatment. A number of mAbs have been developed and approved for use in human anti-cancer therapy. Of the mAbs used to target neoplastic cells,

relatively few have been shown to activate C. However, the anti-CD20 mAb Rituxumab is known to activate C on non-Hodgkins lymphoma cells and contribute to cell destruction via CDC (Di Gaetano et al. 2003). Another mAb specific for CD52 (Alemtuzumab) is also dependent on C activation to mediate anti-leukaemia function (Zent et al. 2004). Both mAbs have been approved for *in vivo* use as an adjuvant to conventional therapies e.g. surgery, chemotherapy. In order to fully maximise the potential of mAb immunotherapy, strategies to improve the efficiency and enhance C activation have been sought. Such strategies are outlined in Section 1.10 and include chemical engineering of antibody constructs (Harris et al. 1997), and preferential selection of IgG1 and IgG3 sub-classes in order to maximise C activation (Idusogie et al. 2001). Targeted inhibition of CReg activity on tumour cell surfaces has also been used to improve therapeutic efficacy. Thus, reducing CReg activity *in vitro* using cytokines (Blok et al. 1998), siRNA (Zell et al. 2007), and mAbs were shown to sensitise tumour cell lines to CDC (Harris et al. 1998, Ziller et al. 2005). Therefore, bi-specific mAbs recognising both a tumour antigen and a CReg represent a promising strategy able to simultaneously activate C and sensitise target cells to CDC. Work in this area is ongoing with a number of *in vitro* studies providing promising evidence for enhanced killing of tumour cells. Enhanced C3b deposition and CDC have been observed in a colorectal (Gelderman et al. 2002b), and cervical carcinoma model (Gelderman et al. 2002a) following bi-specific targeting of a tumour antigen and CReg. Additionally, a spheroid model of renal carcinoma metastases showed significant killing and clearance of micrometastases following targeting using a bi-specific mAb against G250 antigen and CD55 (Sier et al. 2004). Evidence to support the use of these Abs *in vivo* is scant, though CDC, along with ADCC, remain promising therapeutic strategies for targeting tumour cells and enhancing immune responses for clearance of tumours in humans. This technique may be used as a standalone therapy or in conjunction with conventional methods such as surgery and chemotherapy to improve treatment efficacy and reduce potential metastases.

7.7. Conclusions

Thus far, strategies to enhance C activation on tumour cells either by mAb targeting or CReg inhibition have been performed on the premise that C contributes to tumour cell clearance and improves patient prognosis. However, more recent data presented by (Markiewski et al. 2008), and in this thesis point to a pro-tumour role for C-activation suggesting that careful consideration and further research is needed to investigate potential pro-tumour consequences resulting in enhanced tumourigenicity and poorer patient prognosis. Such reports are contrary to dogma, challenge current thinking and necessitate a radical rethink and renewed investigation into the role of C in tumour progression in order to acquire a true representation of the double-edged sword role played by the C system in tumour immunology.

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