An Investigation of the Role of Complement in Acute Myeloid Leukaemia

Emma Clare Kempshall

A thesis submitted to Cardiff University in Candidature for the Degree of Doctor of Philosophy

Division of Infection and Immunity
School of Medicine
Heath Park
Cardiff

July 2016

Declaration

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

Signed …….. (candidate) Date……

STATEMENT 1
This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

Signed …….. (candidate) Date ……

STATEMENT 2
This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references. The views expressed are my own.

Signed …….. (candidate) Date ……

STATEMENT 3
I hereby give consent for my thesis, if accepted, to be available online in the University's Open Access repository and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed …… (candidate) Date ……

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS
I hereby give consent for my thesis, if accepted, to be available online in the University's Open Access repository and for inter-library loans **after expiry of a bar on access previously approved by the Academic Standards & Quality Committee.**

Signed …….. (candidate) Date ……

Acknowledgements

First and foremost, I'd like to thank my family. Without Pete's huge support and relentless belief in my abilities, and Amelie and Ned's amazing tolerance of mummy disappearing into her work cave for days on end, this would not have been possible.

I would next like to thank everyone from the Haematology Department at UHW for sharing their wealth of experience and offering excellent advice and guidance. Most importantly, I am hugely grateful to all of the amazing patients who have taught me so much, not just about the terrible conditions they are suffering from, but also about what true strength and resilience is. In addition, they have given me the opportunity to witness the astonishing ability of the immune system to fight cancer. This has provided me with constant inspiration and has hugely influenced both my research and clinical career.

I owe a huge gratitude to CRUK for providing me with so much more than just funding. Critically, the fellowship introduced to Professor Awen Gallimore and gave me the opportunity to pursue my research in the field of Cancer Immunology. It is impossible to convey the huge amount of support Awen has offered me during what has been an extremely challenging PhD process. She has cultivated my interests, buoyed me through the bad times and above all inspired me.

I also greatly appreciate all of the excellent advice and guidance provide by Professors Claire Harris and Paul Morgan. Special thanks goes to Kath Smart and Tim Hughes for all of their help and also to all of the lab members, too numerous to mention, who have aided and encouraged me, it has been a brilliant department to work in.

Finally, to my parents who have been relentlessly supportive and have instilled in me a belief that anything is possible- Dad, you'll be relieved to hear that I am now finally a 'proper' doctor.

Summary

Acute myeloid leukaemia (AML) affects about 3000 people in the UK each year. Chemotherapy leads to complete remission in most patients but, up to 70% relapse. The success of allogeneic stem cell transplant (SCT) demonstrates that donor T cells can eliminate residual tumour through a graft-versus-leukaemia effect however, SCT is only viable in a minority of patients. Responses seen with alternative approaches, such as adoptive immunotherapy with tumour specific T cells or inducing T cells by vaccination, have been disappointing, highlighting a clear need for new immunotherapeutic approaches. Immune responses against leukaemia are inhibited by a range of suppressive mechanisms. Identification of novel therapeutic targets capable of unleashing natural and effective immune responses is critical.

The complement system is an enzyme cascade with diverse effector functions instituted by both the innate and adaptive immune systems. Recent evidence suggests that complement promotes the progression of malignancy. The role of complement in AML is largely unexplored but the limited data available implies that complement could have a critical role in both the initiation and progression of AML.

Sub-lytic complement protects cells against further attack by both lytic doses of complement and other pore-formers including perforin, the cytolytic protein used by both NK and $CD8^+$ T cells. This phenomenon termed complement-induced protection (CIP). Thus cancer cells exposed to sub-lytic complement may be protected from lysis by NK and $CD8^+$ T cells with significant consequences for immune escape. When this hypothesis was tested using an *in vitro* model of NK cell killing, no evidence of protection against NK cell killing was observed despite clear CIP against further complement attack. Despite a strongly protective phenotype, subsequent microarray analysis revealed no genetic signature for CIP indicating that mechanisms underpinning CIP require no changes in gene expression.

A mouse model was then used to test the hypothesis that complement promotes progression of AML. The use of both genetically modified mice lacking various complement components and complement inhibitors in wild-type animals, revealed that complement promotes AML progression. CIP was a potential mechanism by which tumours might escape immune elimination *in vivo*, however, this was only a small component of the almost complete protection observed in C3 deficient mice. Putative mechanisms include inhibition of CD8+ T cells and promoting AML seeding.

Should complement prove to have a key role in human AML, available complement therapeutics could progress rapidly into human studies. These might not only have an independent role in improving spontaneous immune responses, but might also impact on all available or novel immune mediated therapies for AML.

Table of Contents

Table of Figures

List of Abbreviations

7AAD	7-Aminoactinomycin D
Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
Ag	Antigen
AML	Acute myeloid leukaemia
ALL	Acute lymphoblastic leukaemia
APC	Antigen presenting cell

BM	Bone marrow
BSA	Bovine serum albumin
C4bp	C4 binding protein
C5aR	C5a receptor
C5aR2	C5a receptor 2 (previously known as C5L2)
C5b-9	C5b, C6, C7,C8,C9 complex (MAC)
CDC	Complement dependent cytotoxicity
CFSE	Carboxyfluorescein succinimidyl ester
CIP	Complement-induced protection
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CR	Complement receptor
CReg	Complement regulator
Crry	Complement receptor related gene Y
CTL	Cytotoxic T-lymphocyte
CTSL	Cathepsin L
CVF	Cobra venom factor
DAF	Decay accelerating factor
DAPI	4'6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSRed	*Discoma* coral-derived red fluorescent protein
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunospot
E:T	Effector: target
FACS	Fluorescence activated cell sorting
fB/fD/fH/fI	Factor B/D/H/I
FHL-1	Factor H-like protein 1
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
FX-Pro	Kodak FX Pro fluorescence imaging equipment
GITR	Glucocorticoid-inducible tumour necrosis factor receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HET	Heterozygous
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1 protein
HSCT	Haematopoietic stem cell transplant
HPSC	Haematopoietic stem cells
iC3b	Inactive C3b
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFN-α /-β/ -γ	Interferon-alpha/-beta/- gamma
Ig	Immunoglobulin
IH	In house
IL	Interleukin
IP	Intra-peritoneal

IV	Intra-venous
KO	Knock-out
LAA	Leukaemia associated antigen
LFA-1	Lymphocyte function-associated antigen 1
mAb	Monoclonal antibody
MAC	Membrane attack complex
MACS	Magnetic-activated cell sorting
MASP	Mannose associated serine protease
MBL	Mannose binding lectin
3-MCA	3-Methylcholanthrene
MCP	Membrane co-factor protein
MDSC	Myeloid derived suppressor cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MALDI-TOF MS	Matrix-associated laser desorption/ionization time of flight mass spectrometer
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
NK	Natural killer
NS	Not significant
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
Pen/Strep	Penicillin/ Streptomycin
PHA	Phytohaemagglutinin
PKC	Protein kinase C
PML-RARα	Promyelocytic leukaemia- retinoic acid α receptor
PNH	Paroxysmal nocturnal haemoglobinuria
RPMI	RPMI-1640 media
ROS	Reactive oxygen species
SC	Sub-cutaneous
SFC	Spot forming cell
STING	Stimulator of interferon genes
TCC	Terminal complement complex (MAC)
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th	T helper
TIL	Tumour infiltrating lymphocyte
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
WT	Wild Type

Chapter 1- Introduction

1.1 The Immune System and Cancer

1.1.1 The Development of Cancer

The transformation of normal cells into a cancerous growth involves both genetic mutations and epigenetic modifications which lead to the sequential acquisition of biological capabilities ultimately resulting in malignancy. In their seminal review Hannahan and Weinberg described six 'Hallmarks of Cancer' which malignant cells need to acquire during their multi-step development (Hanahan and Weinberg 2000):

- Sustaining proliferative signalling
- Evading growth suppressors
- Resisting cell death
- Enabling replicative immortality
- Inducing angiogenesis
- Activating invasion and metastasis

A recent review of these 'Hallmarks' focused on the developing understanding that tumours are not isolated masses of proliferating cancer cells but evolve within a microenvironment which plays a critical role in tumorigenesis (Hanahan and Weinberg 2011). It was proposed that evading immune destruction should be included as a 'Hallmark of Cancer' (Hanahan and Weinberg 2011). The review also acknowledged two processes underlying the development of biological capabilities i) genomic instability which expedites their acquisition and ii) inflammation which fosters the development of multiple hallmark features (Hanahan and Weinberg 2011). Thus the 'Hallmarks of Cancer' now recognize the dual function of the immune system in both protecting against and promoting cancer development.

1.1.2 The Dual Role of the Immune System in Cancer

Rudolf Virchow first noted the presence leukocytes in neoplastic tissue in 1863 and went on suggest a link between inflammation and cancer (Balkwill and Mantovani 2001). Despite this early insight, it is only in the last 20 to 30 years that our understanding of the role of inflammation in cancer has reached a level where it has begun to have an impact on the prevention and treatment of cancer (Balkwill and Mantovani 2001). Inflammation both promotes initial malignant transformation and subsequent progression of cancer. During chronic inflammation a range of factors are produced which promote malignant growth. These include growth factors, which promote proliferation, survival factors which inhibit cell death, proangiogenic factors which enhance neovascularisation and extracellular matrix-modifying enzymes which facilitate invasion (reviewed in (Grivennikov, Greten et al. 2010). Malignant cells elicit a chronic, lingering immune response that promotes the neoplastic process whilst failing to trigger appropriate danger signals that would result in immune clearance (Balkwill and Mantovani 2001; Coussens and Werb 2002; Bhardwaj 2007; Lin and Karin 2007).

Pathogens trigger danger signals which lead to the clearance of infected cells. In a similar way, acute and brisk inflammation can induce spontaneous or treatment-induced tumour regression (reviewed in (Schreiber, Old et al. 2011)). William Coley first recognized the ability of infection to trigger tumour regression over 100 years ago, this led him to develop the bacterial based 'Coley's toxin' which met with some sporadic success. A refined form of 'Coley's toxin', was still in use until the advent of cytotoxic chemotherapy in the 1940s (Nauts and McLaren 1990).

1.1.3 Cancer Immunosurveillance

Studies of both carcinogen-induced and spontaneous tumours in cancer-prone mice demonstrate that immune effector cells can eliminate malignant cells. Tumours develop more rapidly and occur more frequently in mice lacking a variety of immune cells/ functions (Smyth, Dunn et al. 2006). In humans, data derived from patients undergoing transplant who receive immunosuppressants in order to prevent graft rejection have a 3-fold increased risk of cancer (Vajdic and van

Leeuwen 2009) with similar findings in patients with HIV/AIDS supporting this being due to immune deficiency as opposed to any confounding risk factor (Grulich, van Leeuwen et al. 2007). Many of the cancers observed at higher frequencies in immunocompromised patients, such as Kaposi's sarcoma and non-Hodgkin's lymphoma, have recognised, usually viral, infectious causes. This raises the possibility that the immune system is only capable of controlling cancers via an anti-viral immune response. Whilst other cancers which have no recognised infectious aetiology are also observed at increased frequencies in immunocompromised patients, incidence of all cancer is not increased to a similar degree across the board (Vajdic and van Leeuwen 2009). Whilst this might reflect variability in immunogenicity between different types of malignancy, it could arguably be the result of thus far unrecognised infectious stimuli. There are therefore still some doubts about the validity of the immunosurveilance hypothesis. Even if you accept that the immune system offers a degree of protection against the development of cancer (Dunn, Bruce et al. 2002; Swann and Smyth 2007), the existence of cancer as an identifiable disease, in patients with apparently intact immune systems, demonstrates that tumours are able to overcome this defence.

1.1.4 Cancer Immunoediting

The cancer immunoediting hypothesis describes three distinct phases, elimination, equilibrium and escape (Dunn, Bruce et al. 2002). Whilst immune effectors might be able to eliminate malignant cells, if this process is incomplete a period of equilibrium can occur where disease progression is balanced by the hosts immune response, the inherent genetic instability of malignant cells may result in a mutation which edits the cancers phenotype in a way that allows it to evolve and escape from immune control (Dunn, Old et al. 2004). Escape can be via a range of mechanisms such as the down-regulation of immunogenic antigens (Ags), the release of immunosuppressive cytokines or expression of anti-apoptotic molecules (reviewed in (Khong and Restifo 2002)). The intimate and dynamic interaction between emerging tumours and the host stroma means malignancy evolves under the

constraints of whatever selective pressure is imposed on it by the environment (Loveland and Cebon 2008).

Experimental systems used to demonstrate the role of immunoediting in cancer progression have identified both Natural Killer (NK) cells and T cells as being important in both controlling and sculpting the phenotype of developing tumours.

1.1.5 Natural Killer Cells

NK cells are lymphocytes originally identified by their ability to lyse tumour cells without prior sensitization (Herberman, Nunn et al. 1975; Kiessling, Klein et al. 1975). Although named for this natural ability to kill cells *in vitro,* their activity is also increased by exposure to a range of cytokines. As opposed to B and T cells, which have Ag-specific receptors, NK cells target tumour cells via a range of activating and inhibitory cell surface receptors. Although NK cells have long been considered an important effector arm of the innate immune response, more recent data has shown that some NK subsets can be long-lived and even exhibit recall responses (Foley, Cooley et al. 2012; Foley, Cooley et al. 2012).

NK cells mediate cytotoxicity via several mechanisms, the most studied of which is degranulation. NK cells have large amounts of perforin and granzyme stored in cytotoxic granules which means that, when activated, they are able to rapidly lyse target cells (Bryceson, March et al. 2006).

NK cell degranulation can occur following the interaction of activating cell surface receptors, such as NKG2D, DNAM1 and 2B4, and natural cytotoxicity receptors, NKp30, NKp44 and NKp46, which are counterbalanced against signals from inhibitory receptors all of which bind to major histocompatibility complex (MHC) class 1 molecules on target cells (Bryceson and Long 2008). Degranulation can also be triggered following NK cell activation by Abs bound to target cells. This is mediated through cell surface Fc receptors such as FcγRIIIA and/ or FcγIIC expressed

on NK cells, binding to the Fc portion of immunoglobulin on the surface of target cells (Lanier, Ruitenberg et al. 1988; Morel, Ernst et al. 1999).

In addition to degranulation, NK cell activation can result in target cell lysis by triggering death receptor pathways such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas which induce apoptosis via caspase activation.

NK cells are also a major source of a range of cytokines such as Interferon (IFN)-γ, Tumour necrosis factor (TNF)-α and Granulocyte macrophage colony-stimulating factor (GM-CSF) (Fauriat, Long et al. 2010). The mRNA for these cytokines is present in NK cells and can be readily transcribed upon NK cell activation (Bryceson, March et al. 2006). Cytokine release leads to the recruitment and activation of other immune cells resulting in increased antigen presentation and an improved adaptive immune response (Srivastava, Lee et al. 2013).

NK cells are able to control tumour development in experimental mice (Kim, Iizuka et al. 2000). Tumour infiltration by NK cells is also associated with a favourable prognosis in several human cancers (Coca, Perez-Piqueras et al. 1997). There is however evidence that tumours evolve to escape elimination by NK cells (Groh, Wu et al. 2002) with some having been shown to reversibly down-regulate the activity of NK cells (Fauriat, Just-Landi et al. 2007).

1.1.6 Adaptive Immune Response

The adaptive immune system consists of both B and T cells and is able to recognize Ags and generate both effector responses, capable of eliminating target cells, and specific memory responses that protect against future exposure to the same pathogens.

1.1.6.1 T cells

Many subsets of T cells exist which have distinct functions in both promoting and regulating this immune response. T cells which express the co-receptor CD8 ($CD8^+$ T cells) kill cells recognised to be infected or transformed via the production of the cytolytic protein perforin which forms pores through which inducers of cell death, such as granzymes, are introduced into the target cell.

T cells which express CD4 ($CD4^+$ T cells) can differentiate to either help this cytotoxic response by producing interleukin (IL)-2 and IFN-γ or dampen down the response via the production of IL-10 and transforming growth factor-beta (TGF-β).

Whilst Ag-specific lymphocytes are thought to play an important role in tumour immune surveillance (Galon, Costes et al. 2006), tumour-infiltrating lymphocytes (TILs) are often hypo-functional with T cells specific for tumour Ag co-existing with the tumour rather than eliminating it (reviewed in (Rosenberg 2004)). Tumour specific cytotoxic T lymphocytes (CTLs) can be activated and expanded however, the full effector function seen when they are exposed to tumour Ag *in vitro* is progressively lost *in vivo* (Rosenberg 2004). In a similar way, tumour vaccines are able to induce T cells at high frequencies yet these fail to achieve significant tumour elimination (Rosenberg 2004).

1.1.6.2 Antigen Presenting Cells

The efficacy of the immune response depends on the ability of professional antigen presenting cells (APCs) such as dendritic cells (DCs) to process Ags and present them to $CD4^+$ and $CD8^+$ T cells. There has been a significant drive to use the antigen-presenting properties of DCs in order to elicit potent tumour antigen-specific T-cell-driven immune responses (Anguille, Smits et al. 2014; Datta, Terhune et al. 2014). It is believed that DC therapy has the advantage over more passive immunotherapy strategies in that it aims to 'train' the hosts immune system and hence generate an immunological memory for the cancer (Rescigno, Avogadri et al. 2007; Melero, Gaudernack et al. 2014).

1.1.6.3 B cells

Upon antigen binding to receptor, B cells are triggered to proliferate and differentiate into their effector form, plasma cells. Plasma cells produce and secrete antigen specific antibodies which are essentially soluble forms of the original surface receptor (Murphy, Travers et al.). Whilst both naturally occurring anticancer immune responses and many forms of cancer immunotherapy depend on the activity of T cells, the evidence is scarce for an anticancer role for antibody producing B cells (Hannani, Locher et al. 2014). However a recent paper has demonstrated a role for B cells in allogeneic tumour rejection by triggering the crucial first step in the cellular immune response (Carmi, Spitzer et al. 2015). This response was initiated by naturally occurring Abs binding to tumour Ags and interacting with DCs leading to engulfment and antigen presentation which triggered T cell mediated tumour clearance (Carmi, Spitzer et al. 2015).

1.1.7 Immunosuppressive mechanisms

The adaptive immune system mounts robust responses to non-self Ags however, it also responds to self-Ags which can result in the development of autoimmune disease. The immune system has developed several mechanisms to avoid recognition of self-Ags including clonal deletion during thymic selection and functional inactivation of self-reactive lymphocytes. Despite this, autoreactive T cells are a common occurrence, even in the absence of autoimmune conditions, but are kept in check by peripheral tolerance mechanisms. These are mediated by a range of immunosuppressive cells derived from both the $CD4^+$ T cell and myeloid cell populations which play an important role in down-modulating excessive or inappropriate immune responses, thereby preventing immunopathology and autoimmune disease. Many immune responses to tumour are mediated via self-Ags and the success of these responses are hampered by tumours utilising a range of immune tolerance mechanisms in order to avoid immune mediated clearance (Swann and Smyth 2007).

1.1.7.1 Regulatory T cells

Regulatory T cells (Tregs) are suppressive cells which have a key role in maintaining immune homeostasis and immune tolerance and are also critical to limiting excessive and harmful immune responses (Sakaguchi, Yamaguchi et al. 2008). Tregs act in a range of locations, in multiple disease situations in order to suppress or modulate the function of a wide range of cells (Tang and Bluestone 2008). The suppressive capacity of Tregs utilises multiple contact-dependent and contact-independent mechanisms (Shevach 2006; Vignali 2008).

Tregs account for approximately 10% of peripheral T cells and can be produced in the thymus as a functionally mature suppressive T cell subpopulation or induced in the periphery from naive T cells following Ag exposure (Yamaguchi, Wing et al. 2011). The nomenclature for Tregs has recently changed with tTregs denoting thymus derived Tregs and pTregs implying peripherally-induced Tregs (Abbas, Benoist et al. 2013). Most tTregs express the forkhead/ winged helix transcription factor, Foxp3, which is critical to both their development and their functional capacity (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003). FoxP3 whilst reliable in mice, is less so in humans being both absent from some Treg sub-sets and present on non-Tregs (deLeeuw, Kost et al. 2012; Devaud, Darcy et al. 2014). Most $FoxP3^{+}CD4^{+}$ Tregs express CD25 (IL-2 receptor α-chain (IL-2Rα)) at a high level however, CD25 is also expressed on activated conventional T cells. The phenotype of human Tregs has recently undergone a collaborative review (Santegoets, Dijkgraaf et al. 2015). There are however significant differences in Treg phenotype between both healthy controls and cancer patients and between circulating Tregs and those present within the tumour microenvironment (Bergmann, Strauss et al. 2008; Whiteside, Schuler et al. 2012; Adeegbe and Nishikawa 2013).

Tregs have positive roles in maintaining peripheral tolerance, preventing autoimmune disease and limiting chronic inflammatory disease (Vignali 2008). They also appear to play an important, though perhaps controversial, role in cancer. Studies in most mouse tumour models and many human cancers have revealed an

increased frequency and enhanced suppressive capacity of Tregs (Wolf, Wolf et al. 2003; Strauss, Bergmann et al. 2007; Mougiakakos, Choudhury et al. 2010). Conflicting studies have demonstrated the accumulation of Tregs to be associated with both worse (Curiel, Coukos et al. 2004; Wolf, Wolf et al. 2005; Deng, Zhang et al. 2010) and improved prognosis (Badoual, Hans et al. 2006; Carreras, Lopez-Guillermo et al. 2006; Tzankov, Meier et al. 2008; Farinha, Al-Tourah et al. 2010; Droeser, Zlobec et al. 2012). Despite this apparently contradictory evidence, there remains clear experimental and clinical evidence to support a role for Tregs in suppressing anti-tumour immune responses and thus avoiding immune clearance (Whiteside 2012).

The release of chemokines in the tumour microenvironment results in Treg recruitment to tumour sites (Ondondo, Jones et al. 2013). Whilst Tregs appear to have a role in promoting immune escape via the suppression of immune effector cells, they might also have a critical role in limiting tumour progression associated with inflammation. Tregs alter their phenotype depending on the concomitant inflammatory response (Yamaguchi, Wing et al. 2011). Tregs might thus limit tumour development in cancers associated with increased inflammatory infiltrates by controlling chronic inflammation (Rech, Mick et al. 2010; Ladoire, Martin et al. 2011) resulting in a better prognosis (Salama, Phillips et al. 2009; Frey, Droeser et al. 2010; Ladoire, Martin et al. 2011; Droeser, Zlobec et al. 2012). The diverse phenotype and functional role of Tregs could thus be dependent on both the tumour type and the 'immune signature' it provokes. Therapies which target mechanisms underlying Tregs diverse roles might thus prove more effective than therapy aimed at Treg ablation.

1.1.7.2 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells that have a remarkable ability to suppress T-cell responses (Gabrilovich and Nagaraj 2009). They are a heterogenous population of cells which regulate immune responses in both healthy individuals and in the context of disease. Although first described in the

setting of cancer (Buessow, Paul et al. 1984; Young, Newby et al. 1987), MDSCs also expand during inflammation and infection (Gabrilovich and Nagaraj 2009).

MDSCs are produced in increased numbers by the bone marrow (BM) and spleen of tumour-bearing mice and suppress anti-tumour responses (Gabrilovich and Nagaraj 2009). Increased levels are also found in the peripheral blood of patients with cancer (Almand, Clark et al. 2001). MDSCs suppress $CD8^+$ T cell activity and are one of the mechanisms utilised by tumours to evade immune control, they have also been shown to limit the efficacy of cancer immunotherapy (Serafini, De Santo et al. 2004; Kusmartsev, Nagaraj et al. 2005; Serafini, Borrello et al. 2006; Sica and Bronte 2007; Gabrilovich and Nagaraj 2009). Their activated state is characterized by the production of reactive oxygen and nitrogen species (RONS) and arginase (Gabrilovich and Nagaraj 2009). In addition to suppressing adaptive immune responses they can also act on innate immune responses by modulating cytokine production by macrophages (Sinha, Clements et al. 2007). In addition, MDSCs have a range of non-immunological functions including promoting tumour angiogenesis, invasion and metastasis (reviewed in (Murdoch, Muthana et al. 2008)).

1.2 Acute Myeloid Leukaemia

1.2.1 Incidence, Presentation and Classification

Acute myeloid leukaemia (AML) is a haematological malignancy which affects the myeloid compartment of the haematopoietic system resulting in an excess of immature cells, or 'blasts', which infiltrate the BM resulting in deficiencies of other blood components. AML is the commonest acute leukaemia in adults with an overall incidence of approximately 8 cases per 100,000 population (Statistics 2013). Incidence increases with age meaning that the 1-2 cases per 100,000 adults aged between 20 and 35 years rises to approximately 25 cases per 100,000 adults over 70 years of age, resulting in a median age of onset of 69 years (Statistics 2013).

Patients generally present with symptoms resulting from cytopenias such as fatigue, infection or bleeding. An abnormal full blood count results in the examination of a blood film and the patient undergoing a BM examination in order to confirm the diagnosis. A range of additional investigations such as immunophenotyping, cytogenetics and molecular genetics allow full characterisation of the disease both aiding diagnosis and allowing patients to be designated a risk category.

The World Health Organisation (WHO) classification incorporates clinical features, morphology, immunophenotyping, cytogenetics and molecular genetics in order to divides cases of AML into prognostically valid groups (Swerdlow 2008). Good risk patients are those found to have balanced translocation/inversion such as t(8:21) or inv(16) or normal cytogenetics with a mutated nucleoplasmin 1 (NPM1) gene (but lacking a Flt3-ITD mutation). The adverse risk groups includes those with t(9;11), inv(3) or complex cytogenetics as well as secondary AML (therapy related or post myelodysplasia) (Swerdlow 2008).

A large proportion of patients fall into an intermediate risk group and their outcome is hardest to predict. Being able to risk stratify these patients is an important area of investigation as it might result in major implications to their management by identifying those that are likely to do well from conventional therapy and those who require a more aggressive course of action. There has been significant progress in this area with the discovery of molecular genetic lesions such as FLT3-ITD, NPM1 and CEBPA being found to significantly affect outcome and therefore being included in the WHO classification (Swerdlow 2008).

1.2.2 Management of AML

Despite continuous, large, ongoing clinical trials, the current optimal treatment for AML has not altered substantially over the last 40 years and still involves intensive induction combination chemotherapy followed by further consolidation therapy (Yates, Wallace et al. 1973). Induction chemotherapy results in up to 85% of patients under 60 years of age achieving a complete remission falling to

approximately 50% in patients over 60 years of age. Consolidation therapy with either further chemotherapy or an allogeneic haematopoietic stem cell transplant (HSCT), leads to an overall survival (OS) of 40% in patients under 60 years of age falling to less than 15% in those aged over 60.

Whilst survival rates in patients over 65 years of age have remained unchanged, outcome for patients aged between 25-64 years has improved from around 30% to nearly 40% over the last 10 years (2015). This is likely due to involvement in clinical trials, the ability to identify patients who will benefit from an intensive approach (and those who will not) and improved outcomes from allogeneic transplantation. It is also the case that the aim of therapy can be different in older patients. The best approach in this patients group might involve less toxic treatment that, whilst not curing their leukaemia, may prolong survival and achieve a better quality of life, but which may not impact on 5-year survival rates. There is therefore a need for the development of alternative, more effective, biologically inspired and better tolerated treatment strategies (Rashidi and DiPersio 2016).

Therapy in AML is a dynamic process with patients who fail to achieve an adequate response being considered for a more intensive approach. The ability to measure minimal residual disease (MRD) in AML is an evolving field. However, whilst the value of MRD monitoring in acute promyelocytic leukaemia (APL) is proven and has thus been accepted as standard practise (Grimwade, Jovanovic et al. 2014), its value has yet to be determined for AML. Advances in the ability to detect leukaemia-associated aberrant phenotypes by multiparameter flow cytometry or leukaemia-specific genetic target by quantitative reverse-transcriptase-polymerase-chain-reaction (RT-PCR) has made the detection of MRD possible in the majority of cases of AML. Whilst this is highly predictive of outcome, conclusive evidence of the value of MRD in risk-stratifying treatment in AML is not yet available (Kayser, Schlenk et al. 2015). The current NCRI AML 17 trial aims to determine the impact of MRD monitoring on survival, quality of life and utilisation of resources. The ability of MRD detection to improve outcomes is largely determined by the availability of alternative treatment options for patients found to be MRD positive. Whilst further

intensive chemotherapy or standard allogeneic HSCT remain the only viable treatment options, MRD is perhaps unlikely to live up to its full potential.

Alternative therapies are essential in order to capitalise on these 'early warning' tests and although targeted therapy directed at underlying genetic or epigenetic aberration is an active area of research, it is yet to reap any large benefits. Whilst targeting a mutation might eliminate that clone or sub-clone, the genetic heterogeneity that exists both within an individual patient and between cases of AML limits this approach (Rashidi and DiPersio 2016). Other research aims to circumvent the heterogeneity of AML by targeting AML-niche interactions which have a key role in development, progression, chemotherapy resistance and relapse in AML (Rashidi and DiPersio 2016). A second key area of research which could have a major impact on all patients and all disease subgroups is the development of alternative immunotherapies for AML.

AML is known to affect normal immune function and allogeneic HSCT is a successful and widely used form of immunotherapy however, its high level of toxicity has led to a quest for alternative immune based therapies which offer the activity of HSCT with less toxicity. The use of cytokines, monoclonal antibodies, vaccines and cell therapy are potentially attractive options especially since levels of resistant or recurrent disease can now be identified in most patients before a frank relapse occurs.

1.2.2.1 Allogeneic haematopoeitic stem cell transplantation

AML is the commonest indication for adults undergoing an allogeneic HSCT, where practically all patients, except those in a good risk category, should be offered a transplant in their first complete remission (Sureda, Bader et al. 2015). There is evidence that in older patients, age may be a more important prognostic factor than standard cytogenetic risk so it is possible that even some cytogenetically 'good risk' older patients might benefit from an upfront HSCT (Schoch, Kern et al. 2004).

Standard allogeneic HSCT offers disease control via both a highly intensive conditioning regimen and the immunological control offered by the graft-versus leukaemia effect (Gupta, Tallman et al. 2011). Graft-versus leukaemia (GvL) is thought to result from the donor-derived T cells ability to recognise minor histocompatibility Ags expressed on host leukaemia cells (Weiden, Flournoy et al. 1979; Baron, Maris et al. 2005). Even in patients with unfavourable cytogenetics who have an extremely poor prognosis, a GvL effect can lead to a long-term remission (Tallman, Dewald et al. 2007). HSCT is a high-risk procedure due to the toxicity of the induction regimen, the risks of immunosuppression and the potential to develop both acute and chronic graft-versus host disease. Due to this high level of toxicity, HSCT is reserved for patients who are unlikely to achieve a sustained remission with conventional chemotherapy. It is also traditionally only available for the young and the fit, which in a condition where over 60% of cases occur in patients over the age of 55 years, is a severe limitation (Statistics 2013).

The three main limitations to transplantation are i) drug toxicity, ii) graft-versus host disease (GvHD) and iii) risk of relapse. These factors are inextricably linked, e.g. attempts to limit GvHD by T-cell depleting grafts leads to increased relapse rates- strengthening the case for a graft versus leukaemia (GvL) effect. A highly ablative conditioning regimen might limit the risk of relapse but this will be at the risk of greater upfront toxicity.

In recent years there has been a paradigm shift in the field of transplantation from heavy conditioning, aiming to eliminate residual disease whilst allowing engraftment, to a reduced intensity approach which relies on the therapeutic impact of the GvL effect. Non-myeloablative or reduced-intensity conditioning (RIC) regimens are now routine practise and have hugely extended the age at which an allogeneic HSCT is an option, with patients up to the age of 70 having been shown to benefit. In fact a large retrospective study concluded that age alone should no longer be seen as a contraindication to transplantation (McClune, Weisdorf et al. 2010), an extremely important finding given the demographics of AML. Despite this huge increase in the average age of patients undergoing an allogeneic HSCT, the risk

of death has fallen steeply over recent years, with a greater than 50% reduction in non-relapse mortality reported over the same period (Gooley, Chien et al. 2010). As expected, the lower toxicity of this reduced conditioning is at the expense of an increased relapse risk however, most studies have concluded an overall benefit in selected patients (Russell, Kjeldsen et al. 2015).

The incidence of relapse post RIC allograft is approximately 30%. Treatment of relapse includes removal of immunosuppression, chemotherapy and donor lymphocyte infusions resulting in approximately 30% of patients achieving a further complete remission, however 2yr survival post relapse is just 14% (Schmid, Labopin et al. 2012). RIC allografts are also not an option in patients with active disease, with studies showing 0% survival in this patient group (Shimoni, Hardan et al. 2006). Patients with residual disease only detectable by immunophenotyping also have a higher risk of relapse although cytogenetically detected MRD did not appear to have an impact (Ustun, Wiseman et al. 2013).

Strategies to reduce relapse rates include post-transplant maintenance chemotherapy and the inclusion of targeted mutation inhibitors, immunotoxins or radioantibody therapy (Dohner, Weisdorf et al. 2015). Observations, such as the ability of CMV reactivation to induce anti-leukaemic activity (Elmaagacli, Steckel et al. 2011) and the ability of the donors' killer-cell immunoglobulin-like receptor (KIR) phenotype to limit disease relapse (Cooley, Weisdorf et al. 2014) are being investigated to try and identify other mechanisms of improving outcome. The use of immunotherapy such as monoclonal antibodies, T-cell therapies or vaccination post-transplant, in an attempt to augment the graft-versus leukaemia effect could also have a potential role.

Several studies have revealed evidence of NK dysfunction in patients with AML compared to other disease groups post HSCT. Early NK cell function drops significantly by day 60 having been comparable to other disease groups at day 30 (Pittari, Fregni et al. 2010). During early NK regeneration donor vs. recipient NK cells appear to protect against disease relapse whilst failing to cause GvHD (Ruggeri,

Capanni et al. 2002). Collectively, these data indicate that NK cells can play an important role in eliminating AML and suggests that therapeutic enhancement of NK function in the early period post transplantation could lead to improved relapse risk without an increased risk of GvHD.

1.2.3 Immune System in AML

AML is a disseminated malignancy at diagnosis with no specific tumour-draining lymph nodes and is therefore likely to utilise different immune evasion strategies compared to solid tumours. Whilst allogeneic SCT provides evidence that donor immune cells can control AML, there is also evidence that the patients' own immune system can also mount an active response (Figure 1.1). Spontaneous remissions, whilst being a well-documented phenomenon in cancers such as renal cell carcinoma or malignant melanoma, are also very occasionally observed in AML (Mitterbauer, Fritzer-Szekeres et al. 1996; Muller, Trepel et al. 2004; Daccache, Kizhakekuttu et al. 2007; Trof, Beishuizen et al. 2007; Burger, Velev et al. 2010). The term 'spontaneous' is perhaps misleading, as remission is generally preceded by an episode of severe sepsis or blood transfusion. Remissions are thought to be due to an overwhelming response to sepsis resulting in raised levels of TNF-α, INF-γ and IL-2 and activation of NK cells, CTLs and macrophages (Trof, Beishuizen et al. 2007). Understanding the mechanisms underlying the ability of patients' own immune systems to eliminate AML is key to the development of successful immunotherapy for AML.

In contrast to solid tumours, such as melanoma or lung carcinoma, in which complex genomic alterations give rise to a range of immunogenic neoantigens, exome and genome sequencing in AML has not only demonstrated a low mutational burden but most leukaemia-specific chromosomal translocations fail to give rise to immunogenic neoantigens (Anguille, Van Tendeloo et al. 2012). This not only makes it difficult for the immune system to recognize and eliminate AML blasts but also results in fewer leukaemia specific Ags (LSA) being available for discovery

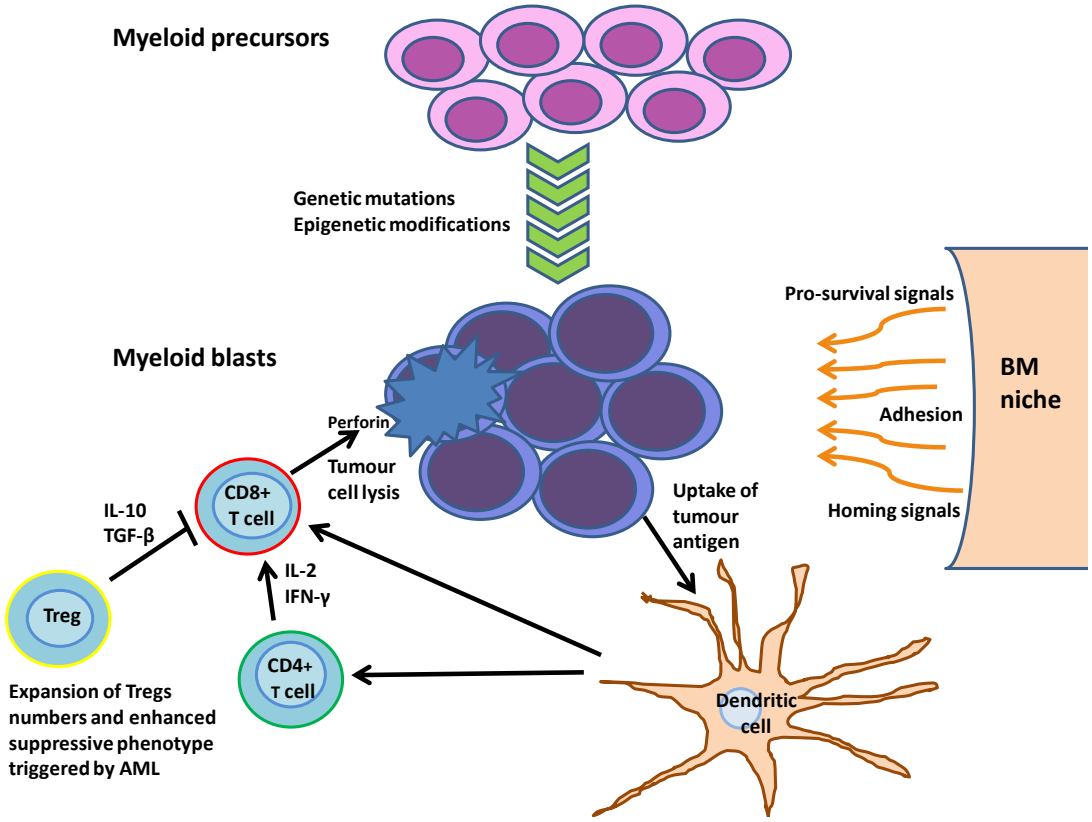


Figure 1.1: Interactions of AML with Immune Cells. A range of genetic mutations and epigenetic mutations result in myeloid precursor cells being transformed into myeloid blast. These can be recognised and killed by the effector arm of the immune system via the presentation of tumour Ags to T cells. However AML has developed a range of mechanisms to avoid immune elimination.

and targeting (Mardis, Ding et al. 2009; 2013). Myeloid cells, such as monocytes, macrophages and myeloid derived suppressor cells (MDSCs), are important to the development of malignancy and can both promote and inhibit tumour growth (Sica and Bronte 2007; Allavena, Sica et al. 2008). In the setting of AML where malignant cells have arisen in the myeloid compartment, functions of myeloid cells could be distinct from other malignant conditions. For example, myeloid blasts themselves have immunosuppressive properties being able to directly suppress T-cell proliferation (Curti, Aluigi et al. 2007; Mussai, De Santo et al. 2013).

1.2.3.1 Natural Killer cells in AML

In vitro killing assays have shown that whilst in other diseases, such as ALL, only a minority of cells are killed by alloreactive NK clones, 100% of acute and chronic myeloid leukaemias are susceptible to lysis by NK cells (Ruggeri, Capanni et al. 1999). *In vivo,* NK success is best demonstrated by the success of allogeneic HSCT in AML where donor-versus-recipient NK cell alloreactivity not only reduces the risk of relapse but also leads to better engraftment and protection from GvHD (Ruggeri, Capanni et al. 2002; Ruggeri, Mancusi et al. 2007). However, AML appears to use a variety of mechanisms to avoid NK clearance *in vivo*.

NK cells from AML patients have an altered NCR profile with down-regulated surface expression of NKp30, NKp44 and NKp46 leading to impaired NK cell-mediated cytotoxicity (Costello, Sivori et al. 2002; Fauriat, Just-Landi et al. 2007; Szczepanski, Szajnik et al. 2010). NK cells from patients with AML are more likely to have an unfavourable KIR phenotype (Verheyden, Bernier et al. 2004), high CD139 expression (Baessler, Charton et al. 2010) and reduced IFN-γ secretion capacity (Tajima, Kawatani et al. 1996) all of which promote escape from NK cell immunity.

A range of features of myeloid blasts lead to impaired NK cell mediated cytotoxicity and IFN-γ secretion. These include low or absent AML cell surface ligands for activating receptors (Verheyden and Demanet 2008), shedding of NKG2D-L (Salih, Antropius et al. 2003; Diermayr, Himmelreich et al. 2008), expression of surface and

soluble GITRL (Baessler, Krusch et al. 2009), increased CD137L expression (Baessler, Charton et al. 2010), high CD200 expression (Coles, Wang et al. 2011) and secretion of soluble immunosuppressive factors such as TGF-β and IL-2R (Lim, Worman et al. 1991; Bergmann, Schui et al. 1995). AML cells also have defects in both their intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic mechanisms which could result in a failure to trigger NK mediated killing (Schimmer, Pedersen et al. 2003; Del Poeta, Bruno et al. 2008; Ragusa, Avola et al. 2010). AML is also resistant to TRAIL and Fas-mediated killing due to both low receptor expression (Iijima, Miyamura et al. 1997; Min, Lee et al. 2004; Tourneur, Delluc et al. 2004) and high decoy receptor expression (Riccioni, Pasquini et al. 2005; Chamuleau, Ossenkoppele et al. 2011).

1.2.3.2 Adaptive Immune Responses to AML

Despite an increased absolute number of peripheral blood T cells in AML patients compared to healthy controls, AML uses several host-derived mechanisms in order to evade immune-mediated clearance. The disseminated nature of AML is perhaps key to its failure to trigger a host type I IFN response (Curran, Chen et al. 2016). This is in contrast to the essential role played by type I IFN in generating adaptive immune responses against solid tumours (Dunn, Bruce et al. 2005; Gajewski 2007; Diamond, Kinder et al. 2011; Fuertes, Kacha et al. 2011). The absence of a type I IFN response in AML results in a T-cell tolerant state with failed DC maturation and deletion of AML Ag-specific T cells (Curran, Chen et al. 2016). Induction of type I IFN and other inflammatory cytokines through activation of the stimulator of IFN genes (STING) pathway resulted in a potent leukaemia-specific immune response and prolonged survival (Curran, Chen et al. 2016).

Gene expression profiling in AML reveals aberrant T-cell activation patterns which result in a failure to form effective immune synapses with AML blasts (Le Dieu, Taussig et al. 2009). Myeloid blasts have been shown to express negative co-stimulatory ligands such as programmed death-ligand 1 (PD-L1) and galectin 9 (Gal-9) (Zhang, Gajewski et al. 2009; Zhou, Munger et al. 2011). PD-L1 functions by

binding to PD-1 expressed on T cells, B cells and NK cells, leading to their deactivation.

Despite AMLs low mutation burden, several leukaemia-associated Ags (LAA), such as proteinase 3 (PR3) and Wilm's tumor-1 (WT-1), have been identified (Scheibenbogen, Letsch et al. 2002; Greiner, Bullinger et al. 2008). Although these LAAs can be recognised by the immune system they are not specific to AML and are also expressed on a range of normal tissues, including the thymus. This means that any high affinity thymocytes that develop against the LAAs are likely to be deleted via central tolerance mechanisms, resulting in an ineffective immune response against leukaemia (Curran, Corrales et al. 2015).

Host DCs also play a critical role in regulating deletional T cell tolerance in AML (Curran, Corrales et al. 2015). DCs engulf AML cells and cross-present AML cell-derived Ags to T cells however, this only results in small numbers of partially dysfunctional Ag-specific T cells (Zhang, Chen et al. 2013; Curran, Corrales et al. 2015). The role of DCs might be particularly important in AML, as due to its disseminated nature, the required levels of danger associated molecular patterns (DAMPs) required to activate DCs in solid tumours, may not be achieved. Targeted activation of innate immunity might thus overcome T cell tolerance in AML (Curran, Corrales et al. 2015).

1.2.3.3 Immunosuppressive Mechanisms in AML (Figure 1.2)

AML induces the expansion of immunoregulatory cells such as Tregs and MDSCs (Wang, Zheng et al. 2005; Zhou, Bucher et al. 2009). This is due to both increased proliferation and the ability of AML cells to trigger the conversion of $CD4^{+}25^{-}$ T cells into Tregs via modulation of tryptophan catabolism (Wang, Zheng et al. 2005; Curti, Aluigi et al. 2007). Tregs accumulate in leukaemia-bearing mice with depletion resulting in enhanced anti-leukaemia T cell responses (Zhou, Bucher et al. 2009). Tregs are both more frequent and have a more suppressive phenotype in patients with AML compared to healthy controls (Szczepanski, Szajnik et al. 2009).

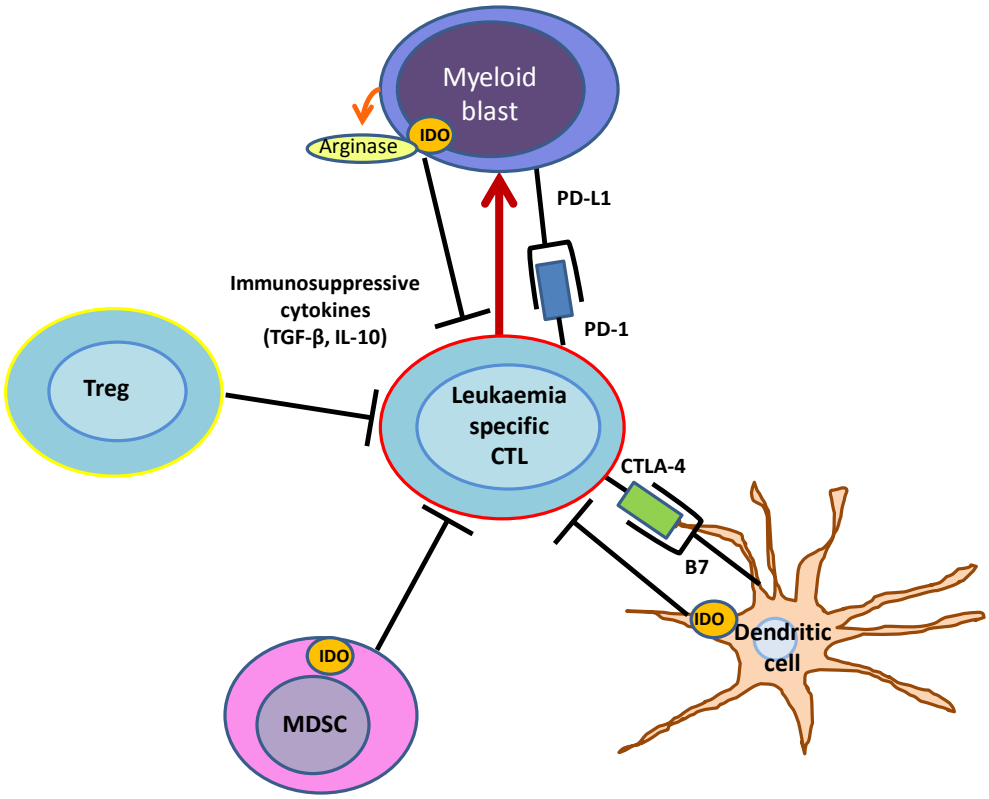


Figure 1.2: Immunosuppressive Mechanisms in AML. AML induces the expansion and activation of immunoregulatory cells such as Tregs and MDSCs. Myeloid blasts themselves also have direct immunosuppressive capabilities directly suppress T cell proliferation via the release of inhibitory enzymes such as arginase or indolamine 2,3-dioxygenase (IDO), which is also released by MDSCs and tumour associated DCs.

Myeloid blasts themselves have direct immunosuppressive capabilities perhaps not surprising, given that they are a malignant expansion of immature myeloid cells, from which MDSCs are also derived. Myeloid blasts directly suppress T cell proliferation via the release of inhibitory enzymes such as arginase or indolamine 2,3-dioxygenase (IDO) which are expressed on blasts taken from AML patients (Curti, Aluigi et al. 2007; Mussai, De Santo et al. 2013). IDO also inhibits T-cell activation and survival and stimulates the conversion of $CD25^-$ cells into $CD25^+$ Tregs (Curti, Trabanelli et al. 2009). High IDO mRNA expression in myeloid blasts correlates with significantly reduced overall and progression free survival (Chamuleau, van de Loosdrecht et al. 2008).

STAT3 is a recognised mediator of cell growth, differentiation and apoptosis and is activated in the majority of AML patients (Benekli, Baumann et al. 2009) being associated with a worse outcome (Benekli, Xia et al. 2002). STAT3 also promotes tumour progression by both modulating $CD4^+$ T cell responses via downregulating production of Th1 cytokines and upregulating production of Th17 cytokines, and by promoting the expansion and immunosuppressive effects of Tregs, MDSCs and tumour-associated macrophages (Zhou, Munger et al. 2011). Studies of the therapeutic effects of STAT3 inhibition in leukaemia reveal that it is these immune-modulatory effects that are key to disease progression (Hossain, Dos Santos et al. 2014).

1.2.4 Non-transplant Immunotherapy in AML

AML is known to alter normal immunological function and evidence from HSCT proves that it is possible to regain immunological control of the disease. The development of immune therapies in AML has lagged behind other solid tumours. This is probably due to the relative success of chemotherapy and HSCT in AML as compared to the very limited therapeutic options previously available in diseases such as malignant melanoma. However, the success of HSCT makes the potential role of alternative immune modulated therapies in AML particularly exciting. A wide range of AML directed immunotherapies are being explored which aim to

deliver the disease control offered by HSCT without the related toxicities. Treatments such as cytokine therapy, monoclonal antibodies, vaccination and cell therapies have met with varying levels of success.

1.2.4.1 Cytokine therapy in AML

As discussed above, myeloid blasts directly impair T-cell and NK cell function resulting in a failure to control leukaemia. Various exogenous cytokines have been investigated in a bid to restore T cell and NK cell function.

Interferon-α (IFN-α) acts via a wide range of mechanisms, involving dendritic cells, monocytes T-cells and NK cells, in order to stimulate the immune system. Monocytes, activated by IFN-α, are able to inhibit malignant growth and induce apoptosis of tumour cells (Bekisz, Sato et al. 2013). IFN-α also has direct inhibitory effects on leukaemia cells (Anguille, Lion et al. 2011). The use of IFN-α has been explored for induction, post-remission and relapse therapy for AML but has failed to demonstrate any clinical benefit.

IL-2 is produced by T cells and activates both T and NK cells as well as stimulating cytokine production and triggering cell cycle proliferation. Whilst IL-2 can stimulate tumour-infiltrating lympohocytes (TILs) (Ettinghausen, Lipford et al. 1985), its effect is dose dependent with low doses both enhancing and suppressing the immune system (Alatrash, Jakher et al. 2013). Most trials of IL-2 occurred in a post-remission setting and meta-analysis has failed to demonstrate any leukaemia-free or OS benefit (Buyse, Squifflet et al. 2011). This failure is thought to be due to either the induction of negative feedback mechanisms (Acuto, Di Bartolo et al. 2008) or the induction of regulatory T-cells (Ustun, Miller et al. 2011). The presence of multiple compensatory mechanisms and feedback loops perhaps makes the failure of cytokine monotherapy unsurprising; combined approaches might prove more effective.

In a similar way to MDSCs, malignant myeloid cells produce ROS which create an arginase-dependent immunosuppressive environment (Mussai, De Santo et al. 2013). Histamine dihydrochloride inhibits the formation of ROS thus restoring the ability of IL-2 to activate T and NK cells (Romero, Thoren et al. 2009). A phase 3 trial of post-remission therapy with IL-2 and histamine dihydrochloride led to an improved 3 year leukaemia-free survival of 40% compared with 26% in controls (Brune and Hellstrand 1996; Brune, Castaigne et al. 2006), however this did not translate to any differences in OS (Yang and Perry 2011). Recent data suggests that both the dynamics of NK cell subsets and NCR expression, and the use of T cell biomarkers that detect the re-distribution of CTL subsets towards a tumour- killing phenotype, could be used to predict clinical outcomes and hence identify patients who would benefit from maintenance therapy with IL-2 and histamine dihydrochloride (Martner, Rydstrom et al. 2016; Sander, Rydstrom et al. 2016).

1.2.4.2 Antibody therapy in AML

Antibody (Ab) therapy in AML is an extremely active area of research with several Ags having been identified for targeting (reviewed in (Gasiorowski, Clark et al. 2014)). CD33 is a transmembrane receptor which although widely expressed on cells of myeloid lineage is also occasionally expressed on activated T and NK cells (Hernandez-Caselles, Martinez-Esparza et al. 2006). CD33 is expressed by 85-90% of myeloid blasts along with leukaemic stem cells and as such offers an attractive target for monoclonal antibodies (mAb) (Tettamanti, Magnani et al. 2013). Gemtuzumab ozagamicin (GO), is a recombinant humanized CD33 mAb conjugated to the cytotoxic antibiotic calicheamicin. After initially disappointing trial results and toxicity issues, the use of lower or fractionated-dose GO has been better tolerated and has prolonged survival in patients with favourable-risk cytogenetics (Burnett, Hills et al. 2011).

New Ab-drug conjugates such as SGN-CD33A, an anti-CD33 mAb bound to SGN-1882, a potent DNA cross-linker, are also under investigation (Kung Sutherland, Walter et al. 2013). Upon binding of the mAb to CD33, SGN-1882 is internalised

resulting in cell-cycle arrest and induction of apoptosis. Promising phase 1 trial results showed blast clearance in 47% of poor risk patients at higher dosing levels with minimal toxicity (Stein EM 2014).

Radiolabelled antibodies are also being used in a bid to deliver radiation directly to malignant cells. Due to toxicity, lack of efficacy and practical issues with delivering the therapy, despite more than 20 years of clinical research, none have moved beyond the research setting. It is likely that their most useful role will be in targeting radiation preferentially to haematopoietic tissues in the HSCT setting (Grosso, Hess et al. 2015).

Bi-specific antibodies are composed of 2 different mAbs which bind to 2 distinct targets such as soluble ligands and cell surface receptors in order to mediate an effect. They can function by re-targeting effector cells to disease-associated sites for example bringing a CTL and a tumour cell together in order to enable immune mediated clearance. They work on the basis that specific surface molecules such as CD3 on T lymphocytes or Fc receptors on monocytes, macrophages and NK cells, can trigger leukocytes to either phagocytose or kill tumour cells. Antibodies against CD123, part of the IL-3 receptor which is preferentially expressed on leukaemic stem cells (LSCs) have been combined with a CD3 Ab in order to form a bispecific T-cell engager which aims to clear these leukaemic precursor cells. Both CD123-CD3 and CD3- CD33 dual-affinity antibodies are currently undergoing clinical trials (Dohner, Weisdorf et al. 2015).

1.2.4.3 Vaccination in AML

Active immunisation of high-risk AML patients following HSCT or conventional therapy is an attractive area of investigation. Vaccines aim to stimulate an anti-leukaemic response in order to control residual disease and thus prevent relapse. Peptide vaccines, granulocyte-macrophage-colony-stimulating factor (GM-CSF) vaccines and dendritic cell (DC) vaccines have all been used to target AML.

Wilms tumor 1 (WT1) is the most widely investigated LAA used to stimulate $CD8^+$ CTLs in AML. A phase 1 trial of a HLA-A*0201-restricted WT1 peptide vaccine in twelve AML patients in complete remission post therapy showed a response in five patients with three continuing on the vaccination and remaining in remission 8 years later (Oka, Tsuboi et al. 2004). GM-CSF in conjunction with peptide vaccination has been used to stimulate an immune response. Of seventeen poor risk patients receiving GM-CSF in combination with HLA-A*0201-restricted WT1 peptide vaccine, ten patients developed increased WT1-tetramer-positive T cells and stable disease, but none achieved a complete remission (Keilholz, Letsch et al. 2009).

PR1, a peptide derived from proteinase 3, is commonly expressed in AML. PR1 peptide vaccination has led to some positive results in phase 1 trials with 4 AML patients vaccinated during their first complete remission who all remained in remission for 4 years post vaccination (Qazilbash MH 2004).

DCs are key to priming Ag-specific CTLs; a role which has been exploited in order to develop leukaemic cell-derived DC vaccines. These originate from leukaemia cells induced to differentiate into mature DCs which are able to present LAAs and thus activate naive T cells (Yuan, Song et al. 2012). These have however proved disappointing with the minimal effects observed in a subset of patients failing to achieve any sustained responses (Roddie, Klammer et al. 2006). Monocyte-derived DC vaccines used in combination with WT1 have had slightly more encouraging results, including immune responses and a few molecular responses (Van Tendeloo, Van de Velde et al. 2010). It is therefore possible that better results will be achieved by using HLA matched allogeneic monocyte derived DCs to stimulate CTLs (Grosso, Hess et al. 2015).

1.2.4.4 Immune Checkpoint Inhibitors in AML

TCR/Ag binding has been demonstrated to be insufficient to result in full T cell activation but requires a second co-stimulatory signal from APCs (Jenkins, Pardoll et

al. 1987). The 1st T cell co-stimulatory molecule to be identified was CD28 (Martin, Ledbetter et al. 1986), the CTL antigen 4 (CTLA-4) was subsequently discovered(Brunet, Denizot et al. 1987; Linsley, Brady et al. 1991) but was unexpectedly found to have a suppressive effect on T cell responses (Walunas, Lenschow et al. 1994; Krummel and Allison 1995). Multiple interactions between T cells and APCs known as immune checkpoints, which fine-tune both duration and strength of T cell responses have since been identified and have led to the development of a range of therapeutic checkpoint inhibitors (Topalian, Drake et al. 2015).

CTLA-4 is a cell surface receptor that is upregulated during T cell activation and plays a role in downmodulating normal immune responses thus preventing uncontrolled immune responses. CTLA-4 is a key part of the natural immune response and key to peripheral T cell tolerance (Fife and Bluestone 2008). It is upregulated in chronically stimulated cells and can thus be co-opted by malignancy in order to hinder anti-tumour immune responses.

The CTLA-4 mAb, Ipilimumab, binds to and blocks CTLA-4 thus taking the brakes off CTL activation. It is licensed for use in advanced melanoma and is currently under investigation in clinical trials for a range of malignancies. CTLA-4 polymorphisms influence the incidence of relapse in AML, suggesting a possible role in control of MRD (Perez-Garcia, Brunet et al. 2009). CTLA-4 blockade enhances immune responses against AML (Zhong, Loken et al. 2006). The safety and efficacy of Ipilimumab is currently being assessed in AML patients at relapse and following HSCT (Andersen 2014).

The inhibitory receptor, programmed death 1 (PD-1), is also upregulated on activated T cells. Interactions between PD-1 and PD-L1, expressed on APCs, results in a contraction of normal immune responses by inhibiting T cell proliferation and cytokine production (Dong, Zhu et al. 1999). PD-L1 is also expressed by a range of malignant cells with its expression level correlating with the aggressiveness of

tumours, poor prognosis and increased risk of death (Thompson, Gillett et al. 2004; Hamanishi, Mandai et al. 2007; Nomi, Sho et al. 2007).

CTLs at the sites of AML progression have elevated PD-1 expression and the PD1-PDL1 interaction appears to facilitate Treg induced suppression of CTLs (Zhou, Munger et al. 2010). PD-1 knockout mice are resistant to AML and administering anti-PD-L1 mAb to WT animals increased CTL infiltration at disease sites, reduced AML burden and led to some long-term survivors. An even greater protective effect was observed when anti-PD-L1 mAb was used in combination with Treg depletion (Zhou, Munger et al. 2010).

Responses to both anti-PD-L1 and PD-1 mAbs observed in patients with solid tumours (Brahmer, Tykodi et al. 2012; Topalian, Hodi et al. 2012) have translated into the clinical use of the anti-PD-1 mAb Nivolumab in both advanced melanoma and squamous cell non-small cell lung carcinoma. PD-1 blockade alone or in combination with DC vaccination or lymphodepletion is currently under investigation in AML patients at high risk of relapse. It remains to be seen whether provisional responses observed in a range of haematological malignancies translate into clinical practice (Berger, Rotem-Yehudar et al. 2008).

TIM-3 and LAG-3 are other co-inhibitory receptors which are emerging as potential therapeutic targets (Pardoll 2012). The combined targeting of multiple negative regulatory receptors also offers an exciting opportunity to further improve efficacy with combined PD-1 and CTLA-4 blockade in melanoma proving more effective than either agent alone (Larkin, Chiarion-Sileni et al. 2015).

1.3 The Complement System

Complement was originally described over 120 years ago, as a heat-sensitive factor found in fresh serum which ‘complemented’ the role of specific Ab in causing lysis of bacteria and red blood cells. Complement has since been shown to be both a crucial effector of innate immunity and an important ‘complement’ to the innate

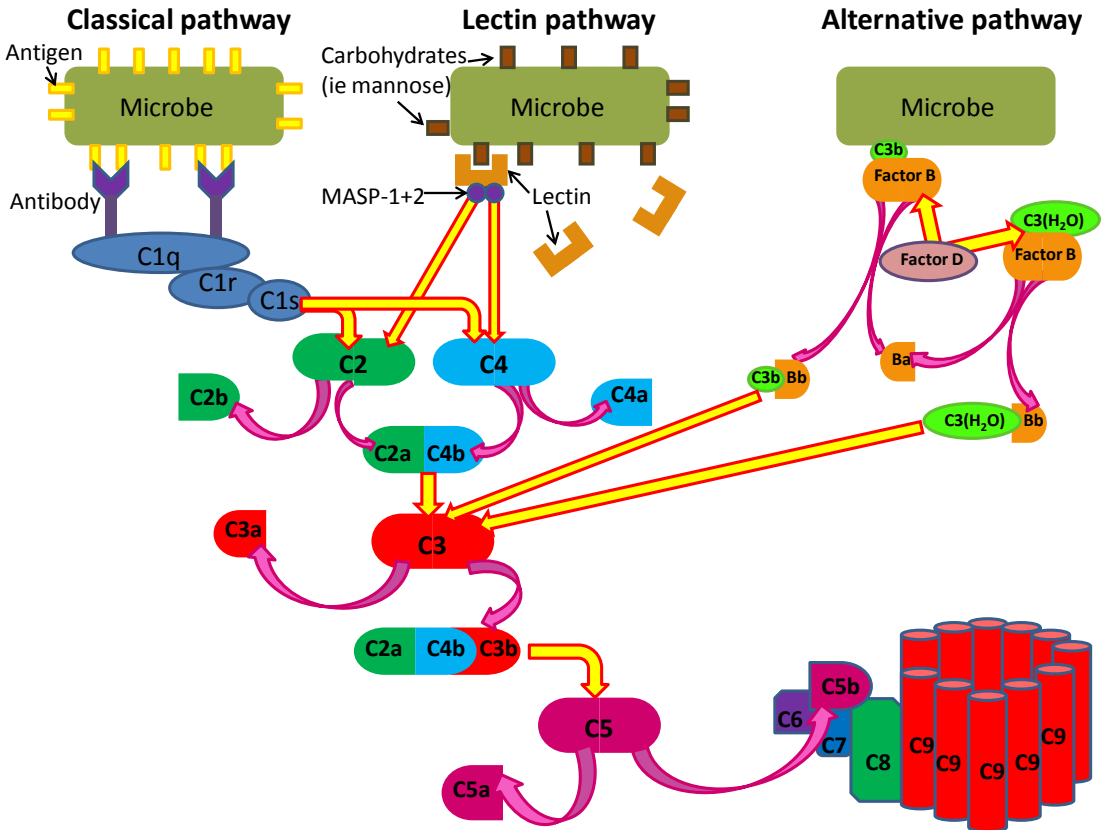


Figure 1.3: The Complement System. An overview of the classical, alternative and lectin complement activation pathways all of which converge on a shared terminal pathway which results in the insertion of a lytic pore into target cell membranes. The classical pathway is triggered by C1q binding antibodies and the lectin pathway by lectin binding mannose residues. In contrast the alternative pathway has no specific activation step, but is based on a 'tickover' mechanism centred around spontaneous low level hydrolysis or protease cleavage at sites of inflammation.

and adaptive immune responses. Complement also assists in the resolution of inflammation via clearance of apoptotic cells and immune complexes (Flierman and Daha 2007; Trouw, Blom et al. 2008). Complement is also involved in a diverse range of other processes such as angiogenesis, mobilization of haematopoietic stem cells (HPSC), coagulation, tissue regeneration and lipid metabolism (Ricklin, Hajishengallis et al. 2010).

Complement is a complex system comprising of more than 50 circulating or cell-surface bound proteins (Pio, Corrales et al. 2014). Three separate activation pathways exist which converge on a shared terminal pathway (Figure 1.3).
Complement proteins either contribute to one or more complement activation pathways or have a regulatory role in order to control autologous complement attack (reviewed in (Walport 2001; Walport 2001)). Complement interacts via a cascade mechanism via which a trigger is amplified in order to culminate in the formation of the membrane attack complex (MAC) constructed from complement proteins C5b, C6, C7, C8 and multiple C9 (C5b-9). MAC is inserted into the plasma membrane of target cells leading to a loss of cell homeostasis and, at an adequate dose, can result in rapid cell death (Morgan and Meri 1994). In addition to its role in direct cell lysis, complement also has a wide range of effects such as enhancing immune responses via the release of anaphylatoxins and opsonisation (Walport 2001). Complement also has a range of non-lethal effects on angiogenesis, coagulation and cell tethering (Langer, Chung et al. 2010; Lupu, Keshari et al. 2014; Hamad, Mitroulis et al. 2015).

Although complement components are primarily synthesized in the liver (Alper, Johnson et al. 1969), numerous other cell types also synthesize complement. Local production of complement proteins appears to have significant effects on tissue homeostasis and immune defence (Morgan and Gasque 1997). Complement production by alternative cells can be significant, for example, bone-marrow derived cells contribute 40% of total C3 in the serum (Morgan and Gasque 1997). C3 is a 185kDa protein comprising two polypeptide chains linked by a disulphide bond (Tack and Prahl 1976). It is present in high concentrations in serum (1-2mg/ml) and,

being common to all three complement activation pathways, is central to the entire complement system. C3 deficiency leads to impaired CDC which results in recurrent bacterial infections (Walport and Lachmann 1984).

Multiple pathways of activation and the catalytic nature of many steps mean that regulation of complement is complex (Morgan and Meri 1994). The complement system is able to attack altered or infected cells whilst limiting damage to host cells by a range of methods. These include tightly controlled activation mechanisms and protection of autologous cells from inappropriate complement attack via the expression of serum and membrane bound complement regulatory proteins (CRegs). Activation is controlled by the use of inactive precursor forms of the complement proteins, or 'zymogens', which require specific activation, allowing complement deposition to be limited to target cells. Upon activation of any of the three complement pathways, inactive precursors are enzymatically cleaved leading to localised deposition of active protein fragments on the target cell. The enzymatic nature of the pathways allows a small trigger to be rapidly amplified to result in a brisk, highly effective complement attack.

A constant low level of activation mediated via the alternative pathway results in the complement system being maintained in a constant state of readiness. This means that when the complement system senses danger in the form of pathogens or altered/damaged self it is able to coordinate a rapid, appropriate cellular innate or adaptive immune response (Kohl 2006). It accomplishes this via the use of danger sensors, such as C1q, which recognise pathogens or altered cells and either activates cell-bound receptors or complement directly by initiating cleavage of C3 and C5 (Kohl 2006). Both the danger signals themselves and the complement cleavage products they produce, function to direct the immune system to an innate or adaptive phenotype via their interactions with cell-bound receptors/regulators (Kohl 2006). Complement is able to discriminate between physiological danger where cells are flagged for enhanced phagocytosis without an inflammatory stimuli and a pathological danger where anaphylatoxins are also increased resulting in an inflammatory response (Kohl 2006).

1.3.1 The Classical Pathway

The classical pathway (Figure 1.4) is triggered by the binding of the complement component C1q to Ab/Ag complexes, formed by the adaptive immune system, which have been deposited on target cells. When an Ag is recognised by specific Abs, an immune complex is formed which allows the Fc portions of IgM and IgG Abs to interact with C1q, the globular domains of the initiator complex C1, triggering focused surface activation of the classical complement pathway (Sim and Reid 1991). C1q can also recognise endogenous ligands such as dying cells, extracellular matrix proteins, and DNA leading to activation of the classical pathway in the absence of Abs (Pio, Corrales et al. 2014).

Binding of C1q leads to activation of the serine protease pro-enzymes C1s and C1r (Kojouharova, Reid et al. 2010). C1s enzyme is able to cleave numerous molecules of the next protein in the cascade, C4, resulting in rapid amplification of the pathway. C4 cleavage results in release of a weak anaphylatoxin, C4a (Hugli, Kawahara et al. 1983) and formation of a larger, highly unstable, C4b component which, if unable to bind rapidly to the target, is rapidly hydrolysed. Surface bound C4b provides a Mg^{2+} dependent binding site for the next component, inactive C2, whose cleavage, which is mediated by activated C1s, releases the C2b component (Nagasawa and Stroud 1977). The resulting C4b2a complex, also known as 'C3 convertase', is also very unstable and, unless it immediately binds to C3, will rapidly lose function stopping the cascade. C3 convertases' short half-life provides the pathway with a high level of self-regulation preventing uncontrolled activation and autologous attack. Non-bound proteins are rapidly hydrolysed, limiting their deposition to the activation site and thus resulting in a highly localised complement attack.

If the classical pathways C3 convertase (C4b2a) manages to bind to C3, this is cleaved resulting in a small anaphylactic component (C3a) and deposition of the C3b with the C4b2a forming a C4b2a3b complex (Kozono, Kinoshita et al. 1990), which

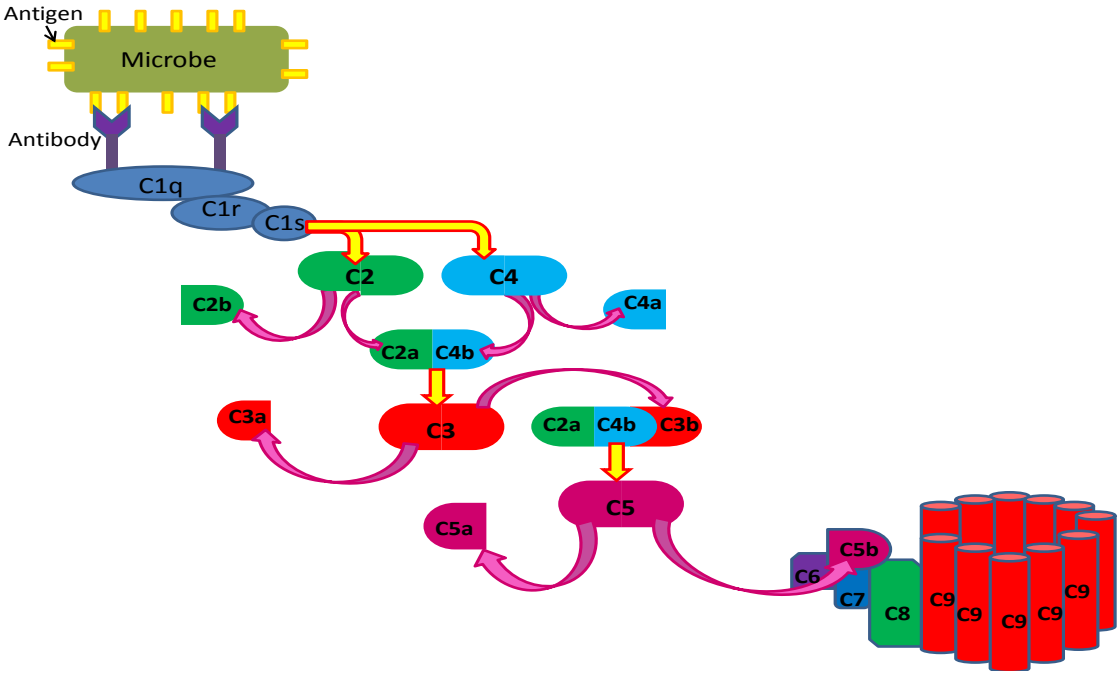


Figure 1.4: Classical Pathway Activation. The classical pathway is triggered by the binding of the complement component C1q to Ab/Ag complexes, formed by the adaptive immune system, which have been deposited on target cells. C1q binding leads to activation of C1s and C1r, C1s in turn cleaves numerous molecules C4, resulting in rapid amplification of the pathway. C4 cleavage results in release of a weak anaphylatoxin, C4a and formation of C4b component which binds to C2a to form 'C3 convertase'. Cleavage of C3 results in an anaphylactic component (C3a) and C3b which forms part of the C5 convertase. C3b deposits on the cell surface also act as a focus for further complement attack.

acts as the C5 convertase of the classical pathway initiating the terminal pathway. C3b deposited on the cell surface also acts as a focus for further complement attack. Approximately 240 C3b molecules are deposited for every original activated C1 complex (Ollert, Kadlec et al. 1994), meaning that, if the tight regulation is overcome, classical pathway activation can result in a huge amplification of the original trigger.

1.3.2 The Lectin Pathway

The lectin pathway (Figure 1.5) closely resembles the classical pathway but is initiated by carbohydrate binding proteins, such as mannose-binding lectin (MBL) and H, L or M- ficolins, recognising arrays of carbohydrates, such as mannose and N-acetyl-glucosamine, found on the surface of pathogenic organisms (Thiel 2007). After binding, MBL interacts with 2 associated serine proteinases, MASP-1 and MASP-2, activation of which results in cleavage of C4 and then C2 resulting in formation of C2bC4a/ C3 convertase, as seen in the classical pathway.

1.3.3 The Alternative Pathway

The alternative pathway (Figure 1.6) differs from the other pathways in that it does not require a specific activation step, such as Ab binding, but is based on a 'tickover' mechanism (Nicol and Lachmann 1973). Spontaneous activation may result from aqueous hydrolysis of C3, this might lead to cleavage resulting in C3b deposition. Either circulating spontaneously hydrolysed C3 ($C3(H_20)$) or C3b deposition on a cell membrane can lead to alternative pathway activation. In addition, any mechanism which results in C3 cleavage and activation, for example enzymes not specific to the complement cascade such as thrombin are able to cleave C3 and trigger the alternative pathway resulting in complement deposition. Activated $C3(H_20)$ can bind to factor B (fB) resulting in formation of a $C3(H_20)B$ complex which is then cleaved by factor D (fD) (Volanakis and Narayana 1996) resulting in the formation of $C3(H_20)Bb$ which is an unstable but active C3 convertase. Although $C3(H_20)Bb$ is able to cleave C3 releasing C3a and forming active C3b, as it is in the fluid phase,

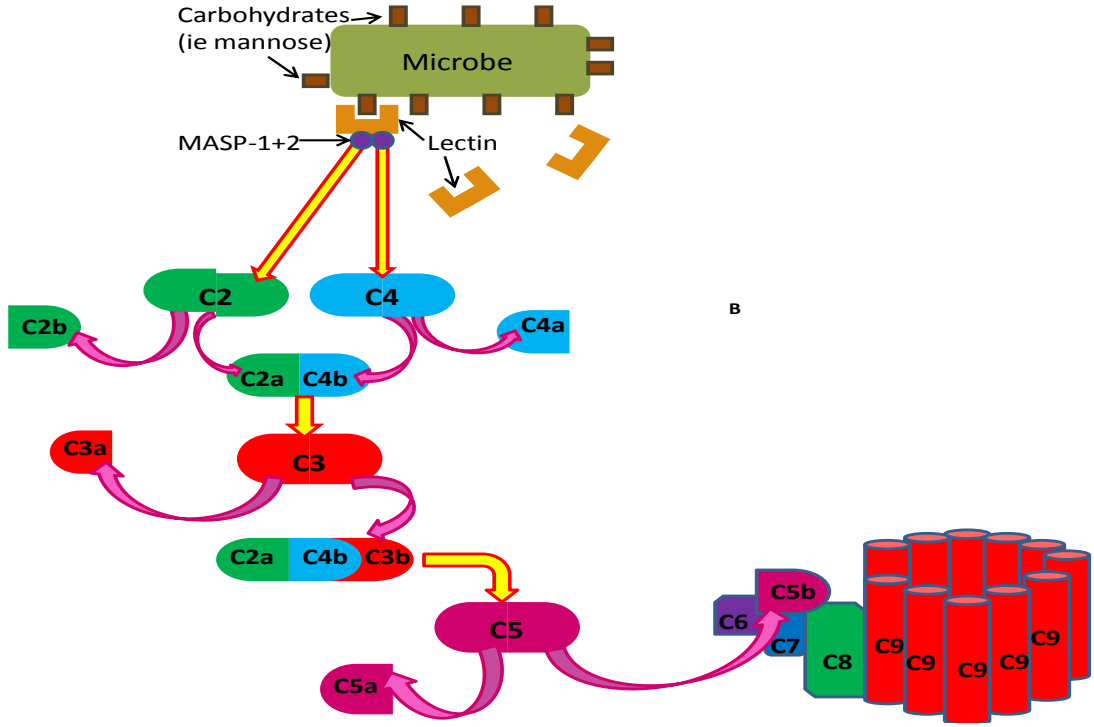


Figure 1.5: Lectin Pathway Activation. The lectin pathway closely resembles the classical pathway but is initiated by carbohydrate binding proteins, such as mannose-binding lectin (MBL) recognising arrays of carbohydrates, such as mannose, on the surface of pathogenic organisms. After binding, MBL activates MASP-1 and MASP-2 which cleave C4 and C2 resulting in the formation of C3 convertase.

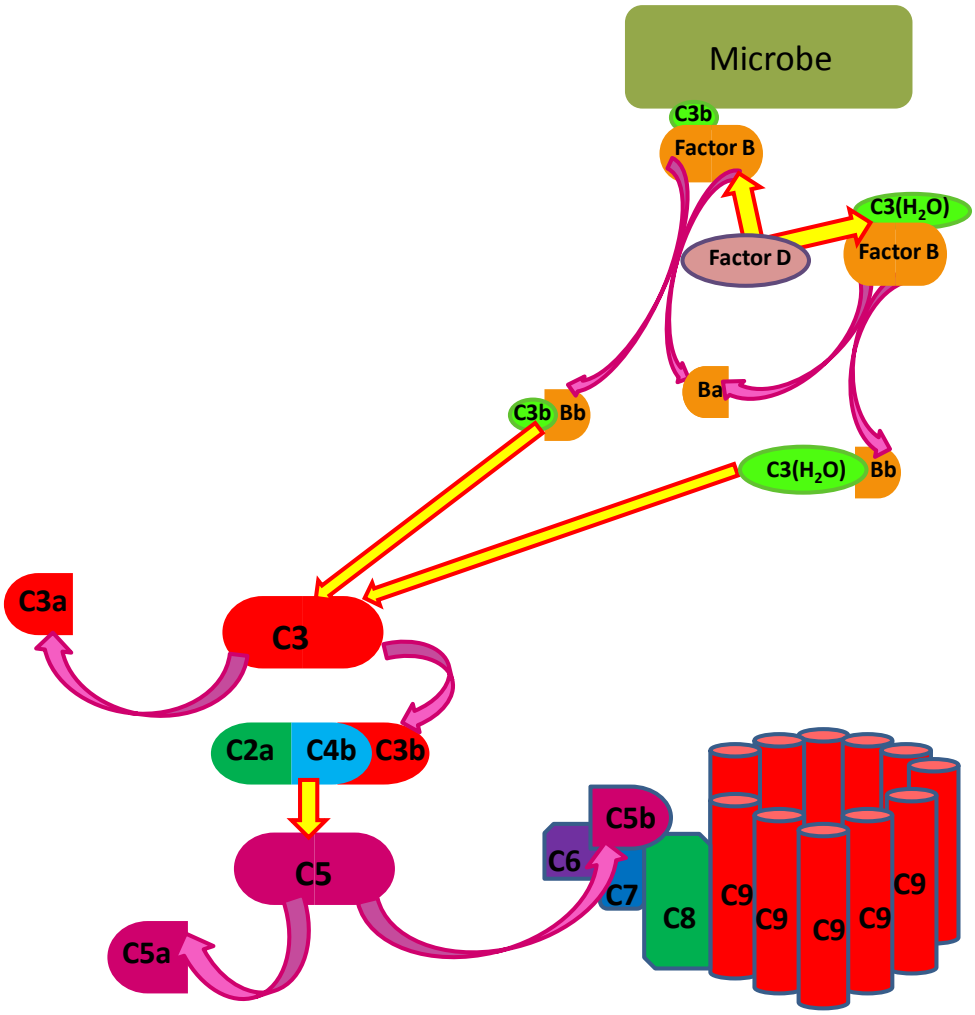


Figure 1.6: Alternative Pathway Activation. In contrast to the classical and lectin pathways, the alternative pathway has no specific activation step. It is based on a 'tickover' mechanism caused by low level C3 hydrolysis or protease cleavage at sites of inflammation leading to spontaneous alternative pathway activation. Activated $C3(H_20)$ and C3b bind to fB forming $C3(H_20)B$ which is then cleaved by fD (Volanakis and Narayana 1996) resulting an unstable but active alternative C3 convertase.

unless the C3b produced is deposited on a cell surface of a target cell, it is rapidly hydrolysed. The pathway is further amplified by the ability of C3b, via a similar interaction with fB then fD, to form alternative C3 convertase. Properties of the surface of a potential target cell determine if the alternative pathway is able to continue or if C3b is cleaved to form surface bound inactive iC3b (Fearon 1979). For example, cell surfaces with low levels of sialic acid less readily bind the alternative pathway regulator factor H (fH) allowing the alternative pathway to be activated on their surface (Fearon 1979). In the presence of surface properdin, a positive regulator of the alternative pathway, the C3bBb is stabilised on the cell's surface, preventing rapid dissociation (Pillemer, Blum et al. 1954) and can cleave numerous C3 molecules resulting in an amplification step. Furthermore, this capacity for amplification is not limited to activation by the alternative pathway but can also be triggered by deposition of surface C3b by the classical and MBL pathways. This is an important feature of the alternative pathway which has been exploited for therapeutic end in several diseases which although triggered by activation of the classical and lectin pathway can be treated by inhibiting the alternative pathway and hence preventing this amplification process (Hu, Holers et al. 2013; Abdel-Magid 2014; Do, Pieramici et al. 2014; Grossman, Hettrick et al. 2016).

1.3.4 Terminal Pathway and MAC Formation

All three pathways converge on a common terminal pathway triggered by C5 convertase which initiates cleavage of C5 resulting in the release of the anaphylactic molecule C5a and exposure of a binding site on C5b (Cooper and Muller-Eberhard 1970). C6 spontaneously binds to C5b followed by C7 which results in a conformation change of the C5b67 complex leading it to be released from the C5 convertase. This release exposes a hydrophobic region in C7 which is able to bind to the target cell membrane (Preissner, Podack et al. 1985). Following binding to C5b67, C8 is inserted into the lipid bi-layer allowing numerous hydrophobic C9 molecules to polymerise into the complex (Tschopp, Podack et al. 1982). The ring structure formed creates a lytic pore through the cell membrane which is abbreviated as C5b-9 and is commonly known as MAC (Figure 1.7).

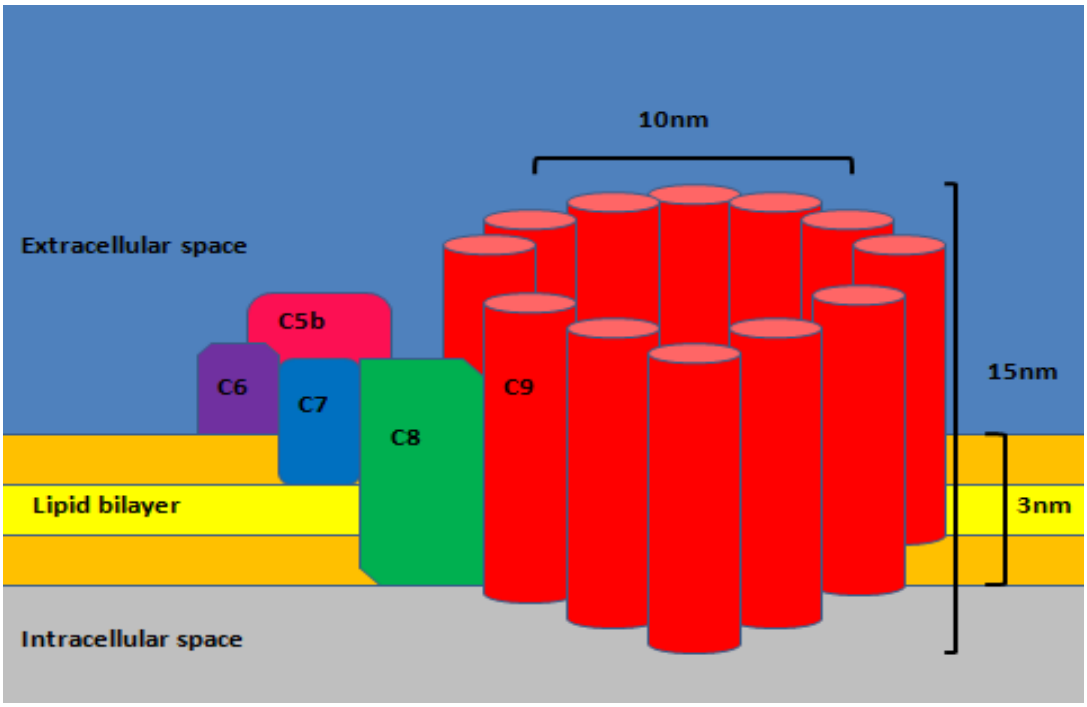


Figure 1.7: The Membrane Attack Complex. C5b, C6, C7, C8 and multiple C9 molecules (C5b-9) form a ring structure which creates a lytic pore through the cell membrane which is commonly known as MAC. The channel formed by the insertion of MAC disturbs the homeostatic capabilities of the cell and in adequate doses can result in cell lysis.

An average of 3-4 C9 molecules typically interact to form MAC, although up to 14 can be involved (Stewart, Monahan et al. 1984). The channel formed by the insertion of MAC disturbs the homeostatic capabilities of the cell and in adequate doses can result in cell lysis (Podack and Tschopp 1982).

Although nucleated cells require the insertion of multiple MAC in order to be lysed, bacteria and eukaryotic cells, such as erythrocytes, are highly susceptible to complement attack and can be lysed by a single MAC complex. Certain pathogens such as gram-positive bacteria, are protected from terminal complement complex (TCC)-mediated lysis via their cell wall architecture by which a thick cell wall prevents MAC from reaching the inner cell membrane (Morgan 1990). MAC is however a major mechanism of lysis for gram-negative bacteria such that individuals who are deficient in components of MAC such as C6 or C7 are highly susceptible to infection with gram negative bacteria such as Neisseria which leads to an increased incidence of meningitis (Morgan and Walport 1991). Pathogens also employ alternative strategies to interfere with TCC assembly.

In addition to its cytolytic role, the presence of MAC results in a range of other important biologically effects including proliferation, resistance to apoptosis, activation of immune cells and protection of the cell from further lytic attack (Cole and Morgan 2003).

1.3.5 Complement Regulators

The regulation of complement activation is a critical homeostatic mechanism which is controlled by a range of soluble and membrane-bound complement regulatory proteins (CRegs) (Table 1.1 and 1.2).

These act via 3 main mechanisms:

i) Blocking the activation cascade by inhibiting protease activity
ii) Facilitating rapid decay and destruction of complement convertases
iii) Preventing MAC formation

mCreg	Expression	Action
Complement receptor type 1 (CR1, CD35)	Erythrocytes, neutrophils, eosinophils, monocytes, follicular dendritic cells, glomerular podocytes, B and some T lymphocytes(Fischer, Appay et al. 1986)	- Cofactor for fI mediated C3b/C4b cleavage (Fearon 1979) - Accelerates decay of classical and alternative convertases (Fearon 1979)
Membrane cofactor protein (MCP, CD46)	Most cells, except erythrocytes (Liszewski, Post et al. 1991)	- Cofactor for fI mediated C3b/C4b cleavage (Liszewski, Post et al. 1991)
Decay-accelerating factor (DAF, CD55)	All blood cells plus most other cells (Medof, Walter et al. 1987)	- Accelerates decay of classical and alternative C3 and C5 convertases (Lublin and Atkinson 1989)
Protectin (CD59)	Most cells (Morgan 1999)	Binds C8 during formation of MAC and prevents insertion of C9 into lipid bilayer (Meri, Morgan et al. 1990)

Table 1.1: Summary of the Expression and Action of Membrane Bound Complement Regulatory Proteins (mCRegs).

In addition to mediating binding of C3 activation products to a range of immune cells, complement receptor 1 (CR1, CD35) also acts as a membrane bound CReg impinging on both the classical and alternative pathways by accelerating the decay of both C3 and C5 convertases. It also acts as a co-factor for factor I (fI) mediated cleavage and thus inactivation of C3b and C4b (Fearon 1980).

Membrane co-factor protein (MCP, CD46) is a co-factor for fI mediated cleavage and inactivation of deposited C3b and C4b (Liszewski, Post et al. 1991). Decay accelerating factor (DAF, CD55), accelerates the decay of both classical and

alternative C3 and C5 convertases (Lublin and Atkinson 1989). The final mCReg which should be mentioned is protectin (CD59) which differs from the others in that it is a specific regulator of the terminal pathway which binds C8 during formation of MAC thus preventing the insertion of C9 into the lipid bilayer (Meri, Morgan et al. 1990).

There are also a range of soluble CRegs (Table 1.2). These include the C1 inhibitor (C1inh) which binds and inactivates C1r, C1s and MASP-2 (Davis, Mejia et al. 2008). FI is a soluble CReg which can only function in conjunction with a range of other CRegs including CR1, MCP, C4bp and fH. Although unable to function alone, fI has a critical role in the regulation of the amplification capacity of the alternative pathway via cleavage and inactivation of C4b and C3b (Sim, Day et al. 1993). It also interacts with CR1 in order to generate C3dg from iC3b. C4 binding protein (C4bp) regulates both the classical and lectin pathways via binding with C4b. It prevents both assembly of C3 convertase and accelerates decay of both this and the C5 convertases (Blom, Villoutreix et al. 2004).

FH and its alternative splice variant fH-like protein 1 (FHL-1) are both alternative pathway regulators. They do this via binding C3b and preventing its association with fB thus reducing the formation of C3 and C5 convertases. They are also able to displace the Bb subunit from formed convertases and act as co-factors for fI mediated cleavage of C3b (Jozsi and Zipfel 2008).

Vitronectin (s-protein) and Clusterin (complement lysis inhibitor, CLI) both inhibit the insertion of MAC into the cell membrane (Podack and Muller-Eberhard 1979; Jenne, Hille et al. 1989). Clusterin has a range of additional roles including modulating cell differentiation and regulating the release of pro-inflammatory cytokines such as TNF-α and IL-6 (Falgarone and Chiocchia 2009).

In addition to the conventional CRegs, somewhat conversely, C8 can also function as an inhibitor of MAC. If soluble C8 binds to C5b67 before it has attached to the

membrane, it prevents the complex from being able to insert into the cell membrane and hence functions as probably the most potent inhibitor of MAC.

sCreg	Action
C1 inhibitor	Inactivates C1r, C1s and MASP-2 (Davis, Mejia et al. 2008)
Factor I	Cleaves and inactivates C4b and C3b (Sim, Day et al. 1993) Generates C3dg from iC3b
C4BP	Binds to C4b and: - Prevents assembly of C3 convertase - Accelerates decay of C3 and C5 convertases - Acts as co-factor to fI. (Blom, Villoutreix et al. 2004)
FH and FHL-1	Binds C3b and: - Competes with fB to reduce formation of C3 and C5 convertases - Displaces Bb subunit from convertases - Acts as cofactor to fI in cleavage of C3b (Jozsi and Zipfel 2008)
Vitronectin	Inhibits MAC insertion into cell membrane (Podack and Muller-Eberhard 1979)
Clusterin	- Inhibits MAC insertion into cell membrane (Jenne and Tschopp 1989) - Modulates cell differentiation - Regulates production of proinflammatory cytokines i.e TNF-α and IL-6 (Falgarone and Chiocchia 2009)
C8	- Binding of soluble C8 to C5b67 before it has bound to the membrane prevents the insertion of MAC into the cell membrane.

Table 1.2: Summary of Action of the Main Soluble Complement Regulatory Proteins (CRegs).

1.3.6 Immune Modulation by Complement

Since complement was first described in the 1800s, its wide-reaching role in the function of multipleareas of the immune system has become ever more apparent. Evidence for its critical position as a 'bridge' between the innate and both the humoral and cellular adaptive immune responses continues to grow (Dunkelberger and Song 2010). Thus complement is not only a chief component of the innate immune system in its own right, but also acts via the release of anaphylatoxins and opsonin deposition to enhance other branches of the immune response (Figure 1.8). Complement has important roles in both inflammation and enhancing adaptive immune response. APC, B, T and NK cell responses are all regulated by an intricate interaction between complement activation products and cell surface receptors (Figure 1.6).

1.3.6.1 Anaphylatoxins

Cleavage of complement proteins results in both the creation of an active component, which goes on to trigger the next step in the cascade, and the release of smaller cleavage products which do not contribute directly to cell lysis. The complement cleavage products, C3a and C5a, act as powerful anaphylatoxins recruiting and activating numerous inflammatory cells to the site of complement activation (Gerard and Gerard 1994). The other complement-produced anaphylatoxins, C4a and Ba, appear to have very weak anaphylatoxic properties and are thus believed to have limited *in vivo* significance (Clark and Klebanoff 1978; Hamuro, Hadding et al. 1978).

C3a and C5a mediate effector mechanisms of target cells and lead to a range of pro-inflammatory immune responses including vasodilation, permeabilisation and smooth muscle contraction (Lambris 1988; Schumacher, Fantone et al. 1991; Ember, Sanderson et al. 1992). C5a and to a lesser extent C3a act as powerful chemoattractants which guide neutrophils, monocytes and macrophages towards sites of complement activation resulting in phagocytosis via binding of their

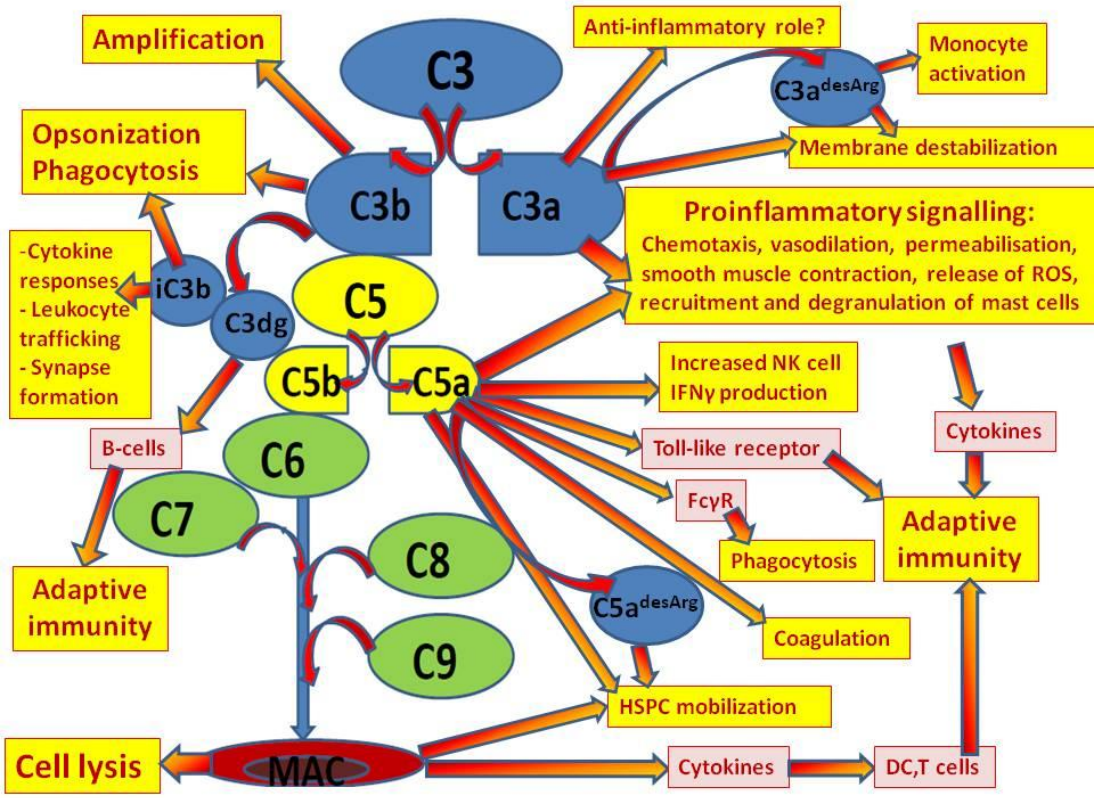


Figure 1.8: Complements Immune Modulatory Network. Complement has important roles in both inflammation and enhancing adaptive immune response acting via the release of anaphylatoxins and opsonin deposition to enhance other branches of the immune response. APC, B, T and NK cell responses are all regulated by an intricate interaction between complement activation products and cell surface receptors. Complement is thus at the centre of a dynamic hub-like network that is involved in both immune surveillance and cell homeostasis via tight connections to other systems.

complement receptors to opsonins, such as C3b, which coat target cells (Haas and van Strijp 2007; van Lookeren Campagne, Wiesmann et al. 2007). Both C3a and C5a are rapidly inactivated by carboxypeptidase-N to their desArg forms, thus avoiding excessive inflammatory activation and damage to host tissues.

C3a

As cleavage of C3 is the central step in the complement system, C3a is found in higher concentration than other complement cleavage products. C3a activates a respiratory burst in macrophages (Murakami, Imamichi et al. 1993) and neutrophils (Elsner, Oppermann et al. 1994) leading to the release of ROS, a major component of inflammatory response. C3a also recruits (Kretzschmar, Jeromin et al. 1993) and activates (el-Lati, Dahinden et al. 1994) mast cells and basophils resulting in the release of vasoactive amines such as histamine. C3a assists migration of cells which express the C3aR (Zwirner, Gotze et al. 1998) whilst other C3 products such as $C3a_{desArg}$ exert their effects in a receptor independent mechanism (Martin, Bock et al. 1997; Zwirner, Gotze et al. 1998).

C3-deficient mice have impaired T cell responses which whilst detrimental in the setting of infection (Kopf, Abel et al. 2002; Nakayama, Kim et al. 2009) have proved advantageous in the setting of autoimmune disease and transplant rejection (Kaya, Afanasyeva et al. 2001; Marsh, Farmer et al. 2001). The ability of DCs to induce potent alloreactive $CD4^+$ T cell responses is dependent on C3a signalling (Sacks 2010).

Complement is traditionally viewed as being a serum-operative system with activation and lysis occurring in the extracellular space however recent data have shown that C3 activation also occurs intracellularly (Liszewski, Kolev et al. 2013). Whilst mainly explored in T cells, intracellular C3a was found to be present in all cell types studied (myeloid, lymphoid, epithelial, endothelial and fibroblasts) suggesting that this could be a general, not a T cell-restricted, phenomenon and could thus have broad physiological significance (Liszewski, Kolev et al. 2013). C3 was

processed into active C3a and C3b by cathepsin L (CTSL), a protease expressed by T cells (Liszewski, Kolev et al. 2013). Resting T cells contain stores of C3 and CTSL as well as substantial amounts of C3a which was shown to be essential for homeostatic T cell survival (Liszewski, Kolev et al. 2013).

C3a also has anti-inflammatory roles, with both C3a and C3adesArg suppressing polyclonal B cell responses (Fischer and Hugli 1997). In sepsis, contrary to the deleterious effects of C5aR signalling, C3aR signalling is protective (Hollmann, Mueller-Ortiz et al. 2008). During acute inflammatory response C3a has also been shown to inhibit mobilization of neutrophils into inflamed tissues (Wu, Brennan et al. 2013). C3a also appears to have anti-inflammatory roles independent of C3aR signalling, such as stimulating secretion of pituitary hormones (Francis, Lewis et al. 2003) and suppression of the pro-inflammatory cytokines TNFα and IL-1 (Takabayashi, Vannier et al. 1996). C3a should thus be considered an immune modulator rather than being considered purely pro-inflammatory (Coulthard and Woodruff 2015).

C5a

Whilst C3a is more plentiful, cleavage of C5 leads to production of the 11kDa C5a protein which, as it is both more resistant to carboxypeptidase-N cleavage and maintains biological activity in its alternative desArg form, is believed to be the most important complement derived anaphylatoxin *in vivo* (Burgi, Brunner et al. 1994). C5a triggers mast cell degranulation leading to the release of histamine, matrix metalloproteinases and inflammatory cytokines (el-Lati, Dahinden et al. 1994; Takafuji, Ishida et al. 2003) implicating it in the pathogenesis of a range of allergic and inflammatory conditions. C5a has chemoattractant effects on a range of cells which express C5aR including neutrophils (Ehrengruber, Geiser et al. 1994), macrophages (Aksamit, Falk et al. 1981), activated B (Ottonello, Corcione et al. 1999) and T cells (Nataf, Davoust et al. 1999). Despite many positive roles, over-production of C5a has been shown to be detrimental in sepsis, mainly due to paralysis of neutrophils (Riedemann, Guo et al. 2003) but also secondary to the

damaging effects of enhanced IFN-γ production by NK cells (Fusakio, Mohammed et al. 2011).

C5a has other diverse roles which strengthen the immune response. Its role in regulating the mobilization of HPSCs from the BM acts to replenish the immune system (Jalili, Shirvaikar et al. 2010). Its ability to induce the expression of tissue factor and plasminogen-activator inhibitor 1 (PA-I1) (Ritis, Doumas et al. 2006; Markiewski, Nilsson et al. 2007), potentiates coagulation and inhibits fibrinolysis thus preventing the dissemination of infection via the bloodstream (Markiewski, Nilsson et al. 2007).

Two receptors have been identified which bind with high affinity to C5a however, the vast majority of its effects seem to be mediated via binding to C5aR whilst the role of the alternative receptor, C5aR2 (previously known as C5L2) remains unclear (Okinaga, Slattery et al. 2003). C5aR2 is often referred to as a 'decoy' receptor which, by binding and removing active complement fragments from the extracellular environment, suppresses the inflammatory nature of C5aR stimulation (Scola, Johswich et al. 2009). Other studies have suggested that C5aR2 does have a functional role in sepsis beyond this with C5aR and C5aR2 being shown to have a synergistic role in detrimental outcome in sepsis (Rittirsch, Flierl et al. 2008). C5aR2 was also demonstrated to have an independent role in release of the pro-inflammatory mediator high mobility group box 1 protein (HMGB1) (Huber-Lang, Sarma et al. 2005).

Both C3a and C5a are produced locally at the APC-T cell interface and determine the outcome of the T cell-APC interaction (Li, Anderson et al. 2008; Strainic, Liu et al. 2008; Peng, Li et al. 2009). C5a promotes the migration of naive and memory B cells (Ottonello, Corcione et al. 1999). The effects of complement on T helper (Th) cells are influenced by both tissue-specific microenvironment and timing meaning that C5aR signalling can be both protective, in an allergen sensitization phase, but destructive once allergic inflammation is established (Kohl, Baelder et al. 2006). Interestingly, C3aR signalling appears to be able to counter the protective effects of

C5aR signalling in an allergen sensitization phase of asthma, contributing to T_h2-dependent airway hyper-reactivity (Drouin, Corry et al. 2002).

The complement system uses both pattern recognition and missing-self recognition strategies in order to trigger its cascade. It is then able to communicate with other biological systems resulting in a coordinated innate immune response (Hajishengallis and Lambris 2010). For example, complement has been shown to cooperate with Toll-like receptors (TLRs) amplifying TLR-induced production of pro-inflammatory cytokines *in vitro* and *in vivo* through C3aR and C5aR signalling (Zhang, Kimura et al. 2007). In this way, concomitant detection of infection by multiple distinct innate defence mechanisms acts to validate the danger and result in a more effective synergistic response.

C5aR has been shown to lower the threshold for Fcγ receptor activation by both up-regulating the expression of activating and down-regulating the expression of inhibitory Fcγ receptors (Shushakova, Skokowa et al. 2002; Kumar, Ali et al. 2006). FcγR activation also enhances the synthesis of C5 (Kumar, Ali et al. 2006) resulting in mutually reinforcing C5a-FcγR crosstalk which by combining phagocytosis with the specificity of IgG antibodies leads to the efficient clearance of pathogens.

Infective, inflammatory or tumour related stimuli differentially regulate expression of a range of receptors for the complement C3 fragments C3a, C5a, C3b, iC3b and C3d which are expressed on NK cells (Min, Liu et al. 2014). Naive NKT and NK cells express high levels of C5aR mRNA, but no protein. During sepsis however, a subset of both NK and NKT cells begin to express C5aR on their surface, suggesting rapid translation of C5aR mRNA into protein on bacterial encounter (Fusakio, Mohammed et al. 2011). C5aR signalling up-regulates NKp46 expression on NK cells resulting in elevated IFN-γ and TNF-α levels which contribute to the detrimental effects of NKT and NK cells during early sepsis (Fusakio, Mohammed et al. 2011). NKT cells also exacerbate sepsis via IL10-mediated inhibition of CD55 expression on neutrophils resulting in enhanced C5a generation (Kim, Oh et al. 2014). Contrary to these findings C5aR signalling has also been shown to impinge on the expression of TRAIL,

a type 1 IFN target gene and pro-apoptotic factor, reducing the production of the type 1 IFNs, IFN-α and IFN-β and protecting against sepsis (Calame, Mueller-Ortiz et al. 2014).

1.3.6.2 Opsonisation

Complement activation results in the deposition of the 'opsonic' complement fragments C3b, C4b and associated inactivation products iC3b, C3d and C3dg. C3b cell surface deposition is essential to all 3 complement activation pathways. Various immune cells also express specific opsonin receptors which recognise deposition of C3b or its breakdown products. Opsonisation with C3b, and to a lesser extent C4b (Clark and Klebanoff 1978), results in enhanced immune responses via adherence of immune cells to complement 'labelled' cells (Fearon 1983; Lambris and Muller-Eberhard 1986) and enhanced phagocytosis. Hence cells which activate complement are not only cleared via MAC deposition but via recruitment of other immune effector cells.

Various immune cell types express cell surface proteins which bind to complement activation fragments these complexes are termed complement receptors 1-4 (CR1-4) (Table 1.3). Complement receptors have a range of functions for example CR1 interacts with both C3b and C4b to promote neutrophil-mediated phagocytosis and in doing so inactivates them, thus blocking further complement activation (Krych-Goldberg and Atkinson 2001). CR1 also cleaves iC3b to C3dg both of which are ligands for CR2 which result in B cell activation and differentiation (Roozendaal and Carroll 2007; Carroll 2008). CR3 and CR4 also bind iC3b promoting phagoctosis whilst CR3 also regulates cytokine responses, leukocyte trafficking and synapse formation (Ricklin, Hajishengallis et al. 2010).

Binding of C3 cleavage products to CR2 and/or CR1 on B cells is essential for Ag-specific Ab responses to both T cell dependent and independent Ags (Thornton, Vetvicka et al. 1996; Da Costa, Brockman et al. 1999; Haas, Hasegawa et al. 2002).

	Expressed	Binds	Functions
CR1 (CD35)	**Erythrocytes** B cells T cells Neutrophils (Wilson, Tedder et al. 1983; Fischer, Appay et al. 1986)	**C3b C4b** (strong) iC3b (weak)	**Co-factor to fI**→ increased C3b cleavage (Fearon 1979) **Removal of immune complexes** by erythrocytes for degradation in spleen (Cornacoff, Hebert et al. 1984) Opsoninsed cells targeted for **endocytosis** (Fearon, Kaneko et al. 1981)
CR2 (CD21)	**B- cells, DCs** (Reynes, Aubert et al. 1985)	**C3dg, C3d** iC3b (Kalli and Fearon 1994)	**B cell** activation and differentiation (Roozendaal and Carroll 2007; Carroll 2008)
CR3 (CD18/ 11b)	**Macrophages**	**iC3b** C4b	Integrin family of **adhesion molecules** Mediates **phagocytosis** of opsonised particles (Hogg 1992) Regulates cytokine responses, leukocyte trafficking and synapse formation (Ricklin, Hajishengallis et al. 2010)
CR4 (CD18/ 11c)	**Neutrophils Platelets Macrophages Monocytes Erythrocytes** (Myones, Dalzell et al. 1988)	iC3b, **C3dg,** C3d (Vik and Fearon 1985)	**Adhesion molecule Enhances localisation** of coated immune complexes and particles

Table 1.3: The Expression, Binding and Activity of Complement Receptors.
Various cell types express cell surface proteins which bind to complement activation fragments these complexes are termed complement receptors (CR) and are numbered CR1-4.

A CR2 targeted Ab was able to suppress 99% of primary B-cell Ab response *in vivo* via blocking binding sites for the opsonins iC3b and C3dg (Heyman, Wiersma et al. 1990). In addition, C3dg binding to CR2 enhances T-cell dependent B cell proliferation (Frade, Crevon et al. 1985; Dempsey, Allison et al. 1996) and promotes B cell survival by mediating Ag-independent signals (Fischer, Goerg et al. 1998). Complement also contributes to the maintenance of B cell memory with C3 breakdown products being shown to bind to CR2 (and to a lesser extent CR1) on follicular DCs within lymphoid follicles (Papamichail, Gutierrez et al. 1975) enhancing the uptake and long-term retention of Ag (Roozendaal and Carroll 2007).

1.3.7 Sub-lytic MAC

MAC deposition results in disruption of the target cell's membrane leading to a loss of homeostasis and at an adequate dose, rapid cell lysis. Anucleated eukaryotic cells, such as erythrocytes, can be lysed by a single MAC complex. More complex nucleated cells require the insertion of multiple MAC and use a variety of mechanisms in order to avoid complement mediated lysis and are thus far more resistant to CDC (Ohanian and Schlager 1981; Koski, Ramm et al. 1983). Low levels of MAC can result in a 'sub-lytic' attack which, although tolerated by the target cell, can lead to a wide range of effects. Factors that may lead to a sub-lytic level of complement attack include low levels of complement activation, the presence of inhibitory molecules and intrinsic membrane repair mechanisms, which can lead to shedding or internalisation of MAC (Koski, Ramm et al. 1983; Campbell and Morgan 1985; Morgan, Dankert et al. 1987).

MAC deposition, even in the absence of cell lysis, results in Ca^{2+} influx and inhibition of cyclic AMP formation (Campbell, Daw et al. 1981; Hallett, Luzio et al. 1981). This immediate influx of Ca^{2+} triggers a wide range of effects and has been shown to have a critical role in protecting nucleated cells from CDC (Morgan and Campbell 1985). Sub-lytic MAC has been shown to drive inflammation and apoptosis via inflammasome activation (Triantafilou, Hughes et al. 2013) independently of C3a and C5a production (Laudisi, Spreafico et al. 2013).

Sub-lytic complement attack triggers protein synthesis and phosphorylation (Reiter, Ciobotariu et al. 1995) and stimulates cell proliferation (Niculescu, Badea et al. 1999). Lipid metabolism has been shown to be affected by the insertion of MAC and plays a key role in cell survival following MAC deposition (Ohanian and Schlager 1981; Papadimitriou, Carney et al. 1991). The finding that protein kinase C (PKC) inhibitors abrogate the protective role of sub-lytic MAC (Kraus and Fishelson 2000) led to the discovery that phosphorylation of ERK mitogen-activating protein kinases (Kraus, Seger et al. 2001), resulted in numerous downstream events including apoptosis, cell proliferation and regulation of gene expression (Pearson, Robinson et al. 2001). Other pathways that have been shown to be activated by sub-lytic MAC include the Ras GTPase family (Niculescu, Badea et al. 1999) which has a role in cell proliferation and the c-Jun family (Rus, Niculescu et al. 1997) which is involved in cell cycle phase transition. Sub-lytic complement attack also leads to *de novo* protein synthesis in both normal (Benzaquen, Nicholson-Weller et al. 1994) and malignant cells (Quattrin, Albini et al. 1992).

Aside from the effects of the actual deposition of terminal MAC, sub-lytic attack also results in the release of complement activation products which have a range of additional effects discussed above.

All of these effects of sub-lytic MAC could be significant when considering the role of complement in tumour development and control. Crucially to the principal hypothesis of this thesis, sub-lytic MAC has also been shown to confer protection against subsequent complement attack (Quattrin, Albini et al. 1992).

1.4 Evidence for a Role for Complement in Cancer

Intimate interactions between emerging tumours and the host stroma mean that tumours develop under a strong selective pressure. Stromal elements such as fibroblasts, macrophages and endothelium facilitate tumour growth by promoting proliferation and neovascularisation and suppressing immune responses (Loveland and Cebon 2008). The diverse roles of complement in regulating mitogenic

signalling pathways, cell proliferation, angiogenesis, resistance to apoptosis, invasion and migration (Rutkowski, Sughrue et al. 2010) could also be critical to the malignant process.

The complement system is activated within the malignant environment with complement deposition and elevated serum levels of complement components observed in a wide range of malignancies (Table 1.4). Interestingly, whilst NK cells and $CD8^+$ T cells play a key role in limiting progression of carcinogen-induced tumours in mice, the complement system promotes progression of these same tumours (Turner 2010). Whether complement deposition reflects an attempt by the immune system to clear tumours or an attempt by the tumour to evade immune clearance remains unclear.

In lung carcinoma C3b deposition is seen in primary tumours (Niehans, Cherwitz et al. 1996) along with high plasma C3c, C4 and levels (Gminski, Mykala-Ciesla et al. 1992) (Corrales, Ajona et al. 2012). Indeed, complement levels have been shown to correlate with tumour size (Nishioka, Kawamura et al. 1976; Guidi, Baroni et al. 1988). Lung cancer cell lines are able to deposit C5b and generate C5a more efficiently than bronchial epithelial cells (Corrales, Ajona et al. 2012). C4d deposits are seen in low grade lymphomas such as follicular and MALT lymphomas but are absent in high grade disease (Bu, Zheng et al. 2007).

MAC deposition is seen in a range of malignancies including breast cancer, where deposits increase following chemo- or radiotherapy and complement activity is also related to disease progression (Niculescu, Rus et al. 1992). Haemolytic complement activity also rises with tumour progression in brain tumours (Matsutani, Suzuki et al. 1984). There is however only limited, and conflicting evidence for the effect of complement activation on prognosis.

High serum MBL- associated serum protease 2 (MASP-2) levels, which is critical to activation of the lectin pathway, is an independent prognostic marker for poor outcome in colorectal cancer (Ytting, Christensen et al. 2005).

Tumour site	Complement abnormalities	Ref
Lung	Increased plasma **C3c, C4** and **C5a** levels. **C3b** and **C5b** (MAC) deposits.	(Gminski, Mykala-Ciesla et al. 1992; Niehans, Cherwitz et al. 1996; Corrales, Ajona et al. 2012)
Multiple myeloma	Defective **C3** activation and deposition	(Zurlo, Schechter et al. 1989)
Low grade lymphoma	Deposition of **C4d** (not seen in high grade lymphomas)	(Bu, Zheng et al. 2007)
CLL	**Low complement** levels	(Schlesinger, Broman et al. 1996) (Fust, Miszlay et al. 1987)
AML	**Classical and alternative pathway activation**	(Minh, Czink et al. 1983)
Neuroblastoma	High **C3** levels, classical pathway activation and haemolytic activity	(Carli, Bucolo et al. 1979) (Gasque, Thomas et al. 1996)
Breast	**C5b-9, C3 and C4** deposits	(Niculescu, Rus et al. 1992)
Ovarian	Elevated **C3a** and **C5b-9** in ascitic fluid	(Bjorge, Hakulinen et al. 2005)
Thyroid	**C3d, C4d** and **C5b-9** complex deposition	(Yamakawa, Yamada et al. 1994) (Lucas, Karlsson-Parra et al. 1996)
Colorectal	Activation of lectin pathway, elevated C3d	(Ytting, Jensenius et al. 2004) (Baatrup, Qvist et al. 1994)
Digestive tract + others	Elevated 'total **haemolytic complement** activity'	(Maness and Orengo 1977)
'Solid tumours'	Elevated **C3c** and C4.	(Guidi, Baroni et al. 1988)

Table 1.4: Complement Activation in Malignancy. Complement deposition and elevated serum levels of complement components are observed in a wide range of malignancy. Whether the presence of complement activation in the setting of cancer reflects an attempt by the immune system to clear malignant cells or evidence that tumours are using complement in order to evade immune clearance, remains unclear.

In contrast, low classical pathway activity at diagnosis has been shown to be highly predictive of short survival in chronic lymphocytic leukaemia (CLL) (Varga, Czink et al. 1995). Low levels of one or more of the complement proteins appear to be a feature of CLL and is related to disease progression with approximately 70% of CLL patients having low levels at diagnosis rising to 100% of advanced stage patients (Fust, Miszlay et al. 1987; Schlesinger, Broman et al. 1996). Interestingly, low complement levels are also observed in healthy first degree relatives of patients with CLL, suggesting that complement deficiency reflects a genetic predisposition to the development of CLL (Schlesinger, Broman et al. 1996).

Despite a longstanding assumption that complement facilitates the elimination of cancer cells (Rutkowski, Sughrue et al. 2010) there is very limited data to support a role for complement in effective clearance of tumours. Complement activation does appear to play a key role in the mechanism of action of anti-cancer mAb immunotherapy, by both triggering direct cell lysis and enhancing Ab-dependent cell mediated cytotoxicity (Clynes, Towers et al. 2000; Dechant, Weisner et al. 2008). *In vivo* studies have however given mixed results with several showing no role for complement in efficacy of mAb therapy (Uchida, Hamaguchi et al. 2004; Minard-Colin, Xiu et al. 2008; Hu, Turner et al. 2009) but others demonstrating a clear beneficial role (Di Gaetano, Cittera et al. 2003; Kennedy, Solga et al. 2003; Imai, Landen et al. 2005; Beurskens, Lindorfer et al. 2012). In human studies, enhancing complement activity appears to improve the efficacy of mAbs. The use of fresh frozen plasma as a source of complement in combination with rituximab in refractory CLL has been remarkably effective (Klepfish, Gilles et al. 2009; Xu, Miao et al. 2011). β-glycan, which has also proved a successful adjuvant to tumour mAb therapy, appears to function by priming the iC3b receptor CR3 on granulocytes and macrophages thus increasing chemotaxis of tumour cells opsonised with iC3b by anti-tumour mAb therapy, (Hong, Hansen et al. 2003). (Allendorf, Yan et al. 2005).

Whilst both *in vitro* and *in vivo* evidence that anti-cancer mAbs activate complement, its role is complex and its contribution to their clinical efficacy is still

rather contentious. As mAbs act via multiple effector mechanisms it is likely that complements role is both agent and clinical context dependent.

In contrast complement appears to have a detrimental effect on the ability of NK activation to improve the efficacy of mAb therapy. C3b inhibits both NK cell activation and Ab-dependent cellular cytotoxicity induced by mAb coated lymphoma cells (Wang, Racila et al. 2008). C3 depletion enhances the ability of mAb coated cells to activate human NK cells and improves the efficacy of mAb therapy in an *in vivo* model (Wang, Veeramani et al. 2009). NK cells also appear to have a critical role in anti-tumour T cell responses triggered by decomplementation in a mouse model of melanoma (Janelle, Langlois et al. 2014). Transient decomplementation with CVF during T cell priming resulted in a more robust and effective $CD8^+$ T cell response which was dependent on increased NK infiltration (Janelle, Langlois et al. 2014).

1.4.1 Complement Regulators in Cancer

mCReg	Cancer type	Findings
↑ **CD59**	Melanoma (Brasoveanu, Altomonte et al. 1996) (Coral, Fonsatti et al. 2000) prostate (Jarvis, Li et al. 1997) neuroblastoma (Chen, Caragine et al. 2000)	Resistance to CDC
	Prostate (Loberg, Wojno et al. 2005)	Increased metastasis
	Prostate (Xu, Jung et al. 2005), colorectal (Watson, Durrant et al. 2006)	Poor prognosis
↑**CD55**	Colorectal (Durrant, Chapman et al. 2003)	Poor prognosis
↓**CD55**	Breast (Madjd, Durrant et al. 2004)	Poor prognosis
↑**CD46**	Breast (Rushmere, Knowlden et al. 2004) (Madjd, Durrant et al. 2005)	Correlates with tumour grade and recurrence

Table 1.5: Membrane Bound Complement Regulatory Protein (mCReg) Expression in Cancer

The expression of mCRegs is the best characterised complement-mediated cancer-associated resistance mechanisms with most cancers over-expressing at least two separate mCRegs. The published associations of up-regulation of mCRegs expression in cancer are summarised in the Table 1.3. A role for mCregs, in protecting against complement-mediated injury is supported by studies using mAbs against complement inhibitors which led to enhanced anti-tumour CDC (Gelderman, Kuppen et al. 2002; Sier, Gelderman et al. 2004; Allendorf, Yan et al. 2005).

The sCRegs also appear to have a role in malignancy. Increased sCRegs expression is seen in a range of malignancies with fI and C4bp being overexpressed in lung cancer cell lines (Okroj, Hsu et al. 2008) and elevated C4bp levels being seen in the plasma of patients with a range of solid tumours (Battistelli, Vittoria et al. 2005). C4bp is also able to bind to a range of ovarian carcinoma cell lines (Holmberg, Blom et al. 2001). C1 inhibitor (Jurianz, Ziegler et al. 2001; Bjorge, Hakulinen et al. 2005) and clusterin (Trougakos and Gonos 2002) may also have a role in protecting cancer cells from complement mediated lysis.

Several studies point to a role for the alternative pathway regulator factor H (fH) and its alternative splice variant fH-like protein 1 (FHL-1) in cancer. A clinically approved immunoassay based on the quantification of FH in the urine exists for the detection of bladder cancer (Kinders, Jones et al. 1998; Cheng, Corey et al. 2005). There is also evidence for a role of FH and/ or FHL-1 in glioblastoma (Junnikkala, Jokiranta et al. 2000), melanoma (Ollert, David et al. 1995), ovarian carcinoma (Junnikkala, Hakulinen et al. 2002) and lung cancer (Ajona, Castano et al. 2004; Ajona, Hsu et al. 2007), where high levels are associated with a poor prognosis (Cui, Chen et al. 2011).

Complement has recently been demonstrated to be an essential component of tumour-promoting inflammation, an effect mediated via fH suppression (Bonavita, Gentile et al. 2015). The long pentraxin PTX53, an essential component of the humoral arm of innate immune system (Bottazzi, Doni et al. 2010), regulates complement by interacting with C1q and FH (Deban, Jarva et al. 2008). PTX53

deficiency results in tumour-promoting inflammation mediated via uncontrolled complement activation (Bonavita, Gentile et al. 2015). This could be relevant to human cancer as PTX53 is epigenetically repressed in a range of human tumours (Bonavita, Gentile et al. 2015). This implies that complement modulation might have a role in preventing malignancies associated with inflammation.

Whilst increased CReg expression appears to be a feature of malignancy, the impact of this is controversial with conflicting studies demonstrating both low and high levels of CReg expression to be associated with disease progression (Koretz, Bruderlein et al. 1993; Hofman, Hsi et al. 1994; Madjd, Durrant et al. 2004) and high levels being linked to decreased survival (Durrant, Chapman et al. 2003; Xu, Jung et al. 2005; Watson, Durrant et al. 2006). Despite these seemingly conflicting findings, it is clear that complement activation and regulation is an active process in the malignant setting and appears to influence the phenotype of tumour cells and hence their capacity to proliferate and survive.

1.4.2 C5a

In 2008 a key paper was published by Markiewski and colleagues which for the first time showed that complement could promote tumour progression by modulating the anti-cancer immune response resulting in a favourable environment for tumour growth (Markiewski, DeAngelis et al. 2008). Using the TC-1 syngeneic model of cervival cancer, they demonstrated that C5a in the tumour microenvironment played a key role by suppressing the anti-tumour $CD8^+$ T cell-mediated response via the recruitment and augmentation of MDSCs (Markiewski, DeAngelis et al. 2008). Regulation of the release of RONS by C5a amplified the suppressive capabilities of MDSCs (Markiewski, DeAngelis et al. 2008).

A C5a mediated tumour promoting effect was also demonstrated in a transgenic model of ovarian cancer, however this effect was not found to be mediated via the immune system but via C5a stimulation of endothelial cells promoting angiogenic activity (Nunez-Cruz, Gimotty et al. 2012). C5a also creates a favourable

microenvironment for progression in lung cancer an effect mediated via both promoting angiogenesis and increasing MDSCs (Corrales, Ajona et al. 2012).

C5a receptor (C5aR) signalling facilitates metastasis in breast cancer by suppressing both $CD8^+$ and $CD4^+$ T cell responses in the lungs via recruitment of MDSCs and regulation of their TGFβ and IL10 production however, no effect on the primary tumour was observed using this model (Vadrevu, Chintala et al. 2014). Other studies in breast cancer have supported a negative role for C5a with C5aR expression being associated with increased size, higher proliferation rates, presence of lymph node metastasis, advanced clinical stages and a worse OS (Imamura, Yamamoto-Ibusuki et al. 2015). Interestingly, examination of primary tumours from patients with C5aR positive metastasis, revealed over half to be C5aR negative supporting a possible role for C5a in tumour progression (Imamura, Yamamoto-Ibusuki et al. 2015).

Contrary to the studies described above, a study pre-dating the Markiewski paper demonstrated that C5a expression by breast cancer clones led to a reduction in tumour growth due to cell cycle interference (Kim, Martin et al. 2005). This finding could be explained by a subsequent study demonstrating the effects of C5a to be dependent on the level of expression (Gunn, Ding et al. 2012). As compared to a complete absence of C5a, high expression levels resulted in more aggressive tumour growth, via recruitment of MDSCs, whilst low levels resulted in a reduction in tumour growth, via enhancing NK, neutrophil and macrophage function (Gunn, Ding et al. 2012).

The majority of evidence thus supports a role for C5a in promoting tumour progression in most cases mediated via inhibiting immune responses but also via promoting angiogenesis, however level of expression might be key.

1.4.3 General Complement Depletion

Using mouse models, it has been shown that whilst NK cells and $CD8^+$ T cells play a key role in limiting progression of carcinogen-induced tumours, complement

actually promotes progression of these same tumours (Turner 2010). Ovarian cancer cell lines grew less well in $C3^{KO}$ and $C3^{HET}$ mice and whilst tumours from $C3^{KO}$ mice were too scarce and small to be fully analysed (Nunez-Cruz, Gimotty et al. 2012), tumours recovered from $C3^{HET}$ mice contained higher frequencies of effector T cells and fewer Tregs compared to WT mice (Nunez-Cruz, Gimotty et al. 2012).

There are several methods of inhibiting complement *in vivo* although they all have limitations. The most widely used, cobra venom factor (CVF), is a structural and functional analogue of C3 which on contact with serum results in the formation of a long-lived C3/C5 convertase which activates C3 and C5 leading to soluble C5b-C9 formation and resulting in the depletion of serum complement activity (Vogel, Smith et al. 1984). CVF is short acting and highly antigenic making data interpretation challenging however it is currently the standard method to which the inhibitory efficacy of experimental anti-complement agents are compared (Morgan and Harris 2003). The transient generalised complement inhibition achieved by this method results in impaired tumour growth in a range of models (Janelle, Langlois et al. 2014; Downs-Canner, Magge et al. 2015).

CVF, humanised CVF and recombinant Staphylococcus aureus superantigen-like protein 7, which directly inhibits complement protein C5, were used to assess complement inhibition in a model of colon cancer (Downs-Canner, Magge et al. 2015). All resulted in a temporary decrease in tumour growth with CVF resulting in increased $CD8^+$ T cells, reduced MDSCs and increased expression of the chemoattractive cytokines CCL5, CXCL10 and CXCL11 (Downs-Canner, Magge et al. 2015).

1.4.4 Complement-Induced Protection

Sub-lytic levels of MAC have been shown to protect cells against further attack by fully lytic doses of complement (Reiter, Ciobotariu et al. 1992). This 'induced protection', which lasted 3-8 hours, was shown to be associated with Ca^{2+} influx and increased protein synthesis and was independent of levels of MAC deposition. Sub-

lytic MAC was shown to almost completely protect cells from a 16-fold increased dose of complement required to lyse >70% of unprotected cells (Marchbank, van den Berg et al. 1997). Differential binding of complement binding Ab and CReg expression did not account for the protected phenotype. Leukaemic cell lines with sub-lytic MAC on their surface rapidly produce a large complement induced protein (L-CIP) (Reiter and Fishelson 1992) which is related to the heat shock protein family and is thought to have a role in tumour escape from CDC (Fishelson, Hochman et al. 2001).

The physiological relevance of this *in vitro*-defined process, termed "induced protection", is unclear although it has been speculated that it prevents lysis of innocent bystander cells at sites of immune activity thereby limiting the extent of collateral damage caused by a given immune response (Reiter, Ciobotariu et al. 1995). Sub-lytic MAC has also been shown to confer resistance to other pore-formers including perforin (Reiter, Ciobotariu et al. 1995), the cytolytic protein used by both NK and $CD8^+$ T cells. In the tumour setting, the consequences of failed CDC and perforin mediated lysis could be highly significant. Malignant cells activate complement and complement proteins are often seen deposited on tumour cells. In contrast to this reflecting elimination of malignant cells, sub-lytic complement attack might protect tumours from clearance by both CDC and NK /$CD8^+$ T cells. Whilst there is no direct evidence to support this hypothesis, it is compatible with many studies demonstrating that complement promotes progression of cancer is mediated by inhibiting $CD8^+$ T cell and NK responses (Turner 2010; Nunez-Cruz, Gimotty et al. 2012; Janelle, Langlois et al. 2014; Downs-Canner, Magge et al. 2015).

Tumour infiltrating lymphocytes (TILs) are often hypo-functional with tumour specific CTLs appearing to co-exist with the tumour without eliminating it (reviewed in (Rosenberg 2004)). Adoptive immunotherapy using *in vitro* expanded tumour specific CTLs has proved disappointing with effector function being progressively lost *in vivo* (Kaech, Hemby et al. 2002; Riddell, Murata et al. 2002; Morris, Hart et al. 2006; Mantovani, Romero et al. 2008; Le Dieu, Taussig et al. 2009). T-cells induced by vaccination have also failed to impact on disease progression

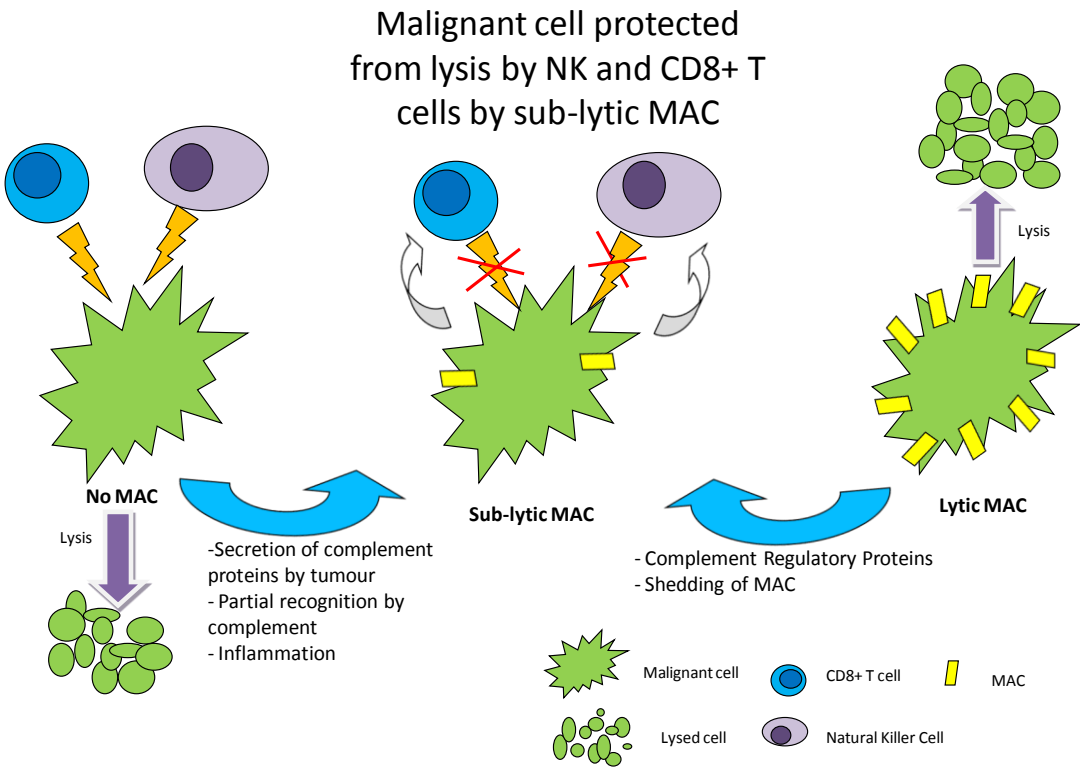


Figure 1.9: Potential mechanisms resulting in induced-protection of malignant cells. Malignant cells could use a range of mechanisms including secretion of complement proteins, expression of complement regulatory proteins and shedding of MAC in order to maintain complement in a constant state of low level activation. In addition low level complement activation may result from malignant cells being partially recognised by the host's immune system. Concomitant low grade infection or inflammation may also result in the correct conditions required for induced-protection to occur.

(Monsurro, Wang et al. 2004) . With these data in mind it is reasonable to expect that compromising the killing capacity of NK cells and CTLs, through a mechanism such as complement-induced protection (CIP), would favour tumour progression (discussed in theoretical paper, (Kempshall, Thebault et al. 2012 see appendix 1) (Figure 1.9).

It is clear that the complement system plays a key but complex role in malignancy which has yet to be fully explored. It is possible that the role of complement could be analogous to the dichotomous role played by the immune system in malignancy with its tumour promoting or protective roles being dependent on both the context and level of activation.

1.5 Complement in AML

There has been very little research into the role of complement in AML. Increased haemolytic activity of both the classical and alternative pathways is observed in patients with AML (Minh, Czink et al. 1983). C4, C3 and fB titres are elevated in patients with AML with serial measurements revealing the highest titres in patients in blastic phase compared to those in remission (Minh, Czink et al. 1983). Myeloid blasts also produce factors which increase complement protein synthesis by monocytes and hepatocytes *in vitro;* an effect mediated by IL-1 and IL-6 (Gyapay, Schmidt et al. 1991; Schmidt, Valay et al. 1995).

More recently a proteomics study identified complement C3f-desArg and its derivative as having a potential role in the diagnosis and MRD assessment of acute leukaemia (Liang, Wang et al. 2010). This study used bead fractionation/MALDI-TOF-MS analysis of sera from patients with AML or ALL (Liang, Wang et al. 2010). Two C3f fragments were identified whose relative intensities gradually decreased as the degree of leukaemic remission deepened (Liang, Wang et al. 2010). Acute promyelocytic leukaemia (APL) is a distinct subtype of AML characterised by the presence of a t(15;17) translocation that results in an abnormal fusion protein termed PML/RARα which is a well characterized and widely used MRD marker

(Grimwade, Jovanovic et al. 2009). Detection of the two C3f fragments was found to be as sensitive a marker for MRD as the PML-RARα fusion protein in identifying MRD positive APL (Liang, Wang et al. 2010). However, in contrast to PML-RARα, MRD detection of these C3 derivatives is applicable across all sub-groups of AML and ALL, thus offering a powerful, widely applicable, diagnostic tool.

These derivatives of C3f have also been identified in other forms of cancer including bladder, prostate, thyroid and breast (Villanueva, Martorella et al. 2006; Profumo, Mangerini et al. 2013). Elevated levels of C3f found in the sera of women affected with the benign condition gross cystic disease of the breast, correlating with the development of breast cancer even 20 years later (Profumo, Mangerini et al. 2013). C3f is cleaved from the α-chain of C3b when it is converted into its inactive form iC3b by fI and H and is rapidly degraded into C3f-desArg (Liang, Wang et al. 2010). FH has been identified as being important in several malignancies including colon, lung, ovarian and bladder cancers (Kinders, Jones et al. 1998; Junnikkala, Hakulinen et al. 2002; Ajona, Castano et al. 2004; Ajona, Hsu et al. 2007; Wilczek, Rzepko et al. 2008). A clinically approved immunoassay used to detect bladder cancer is based on the quantification of both FH and FH-related molecule in the urine (Kinders, Jones et al. 1998; Cheng, Corey et al. 2005).

1.6 Summary

The dual role of the immune system in both fostering and preventing the development of cancer is now widely accepted. The proven ability of the immune system to eliminate leukaemia is demonstrated by the efficacy of allogeneic SCT. Naturally occurring immune responses against leukaemia are inhibited by a range of suppressive mechanisms which both hamper attempts to develop successful immunotherapy and offer novel therapeutic targets aimed at unleashing natural immune responses. Any mechanism which impinges on the ability of the immune system to eliminate leukaemia warrants investigation with the aim of empowering patients' own immune system to control and eliminate leukaemia. The maximal therapeutic benefit is likely to have already been achieved using conventional

combination chemotherapy. Whilst targeting genetic or epigenetic aberrations is an active area of research in AML, this is limited by the genetic heterogeneity that exists both within individual patients and between cases. Research aimed at circumventing this heterogeneity by targeting AML-niche interactions is extremely interesting and has implications on immune interactions. Immune mediated therapies are highly likely to play an important role in future improvements to outcomes in AML.

The complement system has a role as both a crucial effector of innate immunity in its own right and as a mediator of both innate and adaptive immune responses. Complement has been demonstrated to have both inflammatory and anti-inflammatory roles and both protect against and promote the progression of malignancy in both a context and dose dependent manner. The complement system's role in the progression of AML is largely unexplored but limited data implies that it could be critical to both the initiation of leukaemia and its escape from immune control.

1.7 Research Questions

1) **Does exposure to sub-lytic complement protect AML cells from lysis by NK cells *in vitro*?** Published work suggests that sub-lytic complement not only protects cells from lytic attack by complement but also from a range of other pore-formers including perforin. This study used a flow cytometric method to test the hypothesis that sub-lytic complement attack protects AML cells from lysis by NK cells.

2) **Is there a genetic signature for complement-induced protection?** This study compared the genetic signature of untreated AML cells to cells subjected to sub-lytic complement attack.

3) **Does complement affect the progression of AML *in vivo?*** Studies in other malignancies suggest a role for complement in promoting the progression of cancer. This study tested the hypothesis that complement impacts on progression of AML *in vivo*.

4) **Which components of the complement cascade impinge on progression of AML?** Several components of the complement cascade including C3a, C5a, C3b and MAC have the potential to impact on the progression of AML. A combination of mice genetically deficient in various components of the complement system and inhibitors administered to WT mice were used in order to systematically investigate the role of complement in progression of AML.

5) **What are the mechanisms underlying the role of complement in the progression of AML?** Complement has been shown to suppress the $CD8^+$ T cell response against cancer. Experiments were conducted to test the hypothesis that complement promotes progression of AML through effects on the anti-AML T cell response.

Chapter 2- Materials and Methods

2.1 Reagents

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.

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

Freezing solution: 10% DMSO (sigma), 90% FCS (Invitrogen)

Glycine elution buffer: Ultrapure RNAse/DNAse free H_2O (Invitrogen) 0.1M Glycine (Fisher) acidified to pH 2.5 with molecular grade HCl (Sigma)

Low IgG R10 tissue culture media: RPMI-1640 (Gibco) containing 15% heat-inactivated low-bovine IgG FCS (Gibco), 50U penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1% non-essential amino acids

MACS buffer: PBS, 05.% BSA, 5mM EDTA

Phosphate Buffered Saline (PBS) (for use in *in vitro* studies): 8.1mM Na_2HPO_4, 1.5M KH_2PO_4, 137nM NaCl, pH 7.4

PBS-BSA: PBS, 1% Bovine Serum Albumin

PBS-Tween: PBS, 0.1% Tween 20 (Acos Organics)

R10 tissue culture media: RPMI-1640 (Gibco), 10% heat inactivated foetal calf serum (FCS), 50U penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1% non-essential amino acids (Invitrogen)

R+ tissue culture media: RPMI-1640 supplemented with 50U penicillin/streptomycin, 2mM L-glutamine and 1mM sodium pyruvate

SDS-PAGE Loading Buffer (reduced): 0.1M Tris (pH6.8), 10% Glycerol, 2% SDS, 0.01% Bromophenol Blue, 2% β-mercaptoethanol.

SDS-PAGE Running Buffer: 25mM Tris, 191mM glycine, 1% SDS

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

2.2 Antibodies

Antibody	Specificity	Isotype	Application	Dilution	Source
Anti IgM	Goat anti-mouse anti-IgM biotin conj pAb	igG	FACs	10µg/ml	Invitrogen
SA PE	Strepavidin, R-phycoerythrin conj Ab		FACs	2µg/ml	Invitrogen
Anti-C3 HRP	HRP conjugated goat-anti-mouse C3 mAb	IgG	WB	1µg/ml	Abcam
11H9	Rat anti-mouse C3, C3b, iC3b, C3dg mAb	IgG2a	IHC	5µg/ml	Hycult
Isotype control	Rat IgG2a	IgG2a	IHC	5µg/ml	Caltag
BB5.1	Mouse anti-mouse C5 mAb	IgG1	Depletion	10mg/ml	In house
D1.3	Mouse anti-hen egg lysozyme	IgG1	Control for BB5.1	10mg/ml	In house
Anti-rab eryth	Mouse anti-rabbit erythrocyte pAb		Killing assay	2.8mg/ml	In house
AN-18	Rat anti-mouse anti-IFN-γ	Rat IgG1	Capture mAb ELISpot	15µg/ml	Mabtech
R4-6A2	Biotinylated rat anti-mouse IFN-γ	Rat IgG1	Detection mAb ELISpot	1µg/ml	Mabtech
Anti CD8a	PB conjugated anti-mouse CD8a	Mouse IgG2aκ	FACs	5µg/ml	BD Biosciences
Anti	Fitc conjugated	Mouse	FACs	5µg/ml	Biolegend

NK1.1	anti-mouse NK1.1	IgG2aκ			
Isotype control	Mouse IgG2a	IgG2aκ	FACs	5µg/ml	Biolegend
PK-136	Mouse anti-mouse anti-NK-1 mAb	Mouse IgG2aκ	Depletion	5mg/ml	In house
YTS-156	Rat anti-mouse anti-CD8 mAb	Rat IgG2b	Depletion	1mg/ml	In house
YTS-169	Rat anti-mouse anti-CD8 mAb	Rat IgG2b	Depletion	1mg/ml	In house
YTS-191	Rat anti-mouse anti- CD4 mAb	Rat IgG2b	Depletion	1mg/ml	In house
YTA-3	Rat anti-mouse anti CD4 mAb	Rat IgG2b	Depletion	1mg/ml	In house
GL113	Isotype control	Rat IgG2b	Isotype for depletion Ab	1mg/ml	In house

Table 2.1 Antibodies. Summary of the characteristics of antibodies used.

2.3 Cell lines

2.3.1 K562

K562 cells are undifferentiated blasts originally derived from a patient with chronic myeloid leukaemia during blast crisis. K562 has a doubling time of 12 hours, lacks HLA, ALL and thymocyte antigens and is described as being myeloid in origin (Koeffler and Golde 1980). K562 was used in the original studies describing complement-induced protection (Reiter, Ciobotariu et al. 1992; Reiter, Ciobotariu et al. 1995). The K562 cell line is a highly sensitive target for NK cells due to low expression of MHC class I molecules (Drexler 2001).

2.3.2 C1498

C1498 is a mouse acute myeloid leukaemia (AML) cell line which developed spontaneously in C57BL/6 (B6) mice and is classified histologically as myelo-monocytic (Dunham and Stewart 1953). It expresses both a granulocyte marker (gran-1) and 2 macrophage markers (Mac 1, Mac 3) and is uniformly positive for the pan-haematopoietic (CD45) marker Ly 5 (Boyer, Orchard et al. 1995). It is MHC class I positive but class II negative and lacks expression of T cell, pre-B cell and NK cell markers as well as FcR II (Boyer, Orchard et al. 1995). It also expresses both ICAM-1 and ICAM-2 and high levels of LFA-1 (Boyer, Orchard et al. 1995).

C1498FFDsR is a stable transfectant of C1498 that was prepared using a non-viral vector delivery system to achieve expression of both fluorescent *Discoma* coral-derived protein DsRed and firefly luciferase (Sauer, Ericson et al. 2004). C1498FFDsR was initially used in order to accurately detect disease within tissue samples but was subsequently found to allow *in vivo* analysis of disease development using fluorescence imaging equipment.

2.4 Cell Culture

Both the human K562 and murine C1498 cell lines were maintained in R10 tissue culture media. Cells were maintained at a confluence level of between 1 x 10^5 and 1 x 10^6 cells per ml in tissue culture flasks (Nunc) incubated at 37°C, 5% $C0_2$.

2.5 Cell freeze, thaw and storage

For *in vivo* experiments the original vial of C1498FFDSRed cell (henceforth referred to as C1498) supplied by Professor Blazar were bulked up and frozen into multiple aliquots on receipt such that an identical aliquot could be used for each experiment. Cells were re-suspended in freezing solution and aliquoted into 1ml cryovials which were placed into Cryo 1°C Freezing containers (Nalgene) and stored at -80°C. Cells were thawed in a 37°C water bath, re-suspended in 10mls of R10, centrifuged at 450G for 5 minutes before being re-suspended in 10ml of fresh R10.

2.6 Antibody preparation

TG1 is an anti-myeloid cell IgM monoclonal antibody (Ab) originally raised against the glycoprotein fraction of thymocyte membranes(Beverley, Linch et al. 1980). An original batch of TG1 supernatant along with the TG1 secreting cell line was kindly supplied by Dr Alison May (Dept of Haematology, Cardiff University). Following success with early studies using the TG1 supernatant, further supernatant was produced in order to complete the in vitro experiments described in Chapter 3. Supernatant from anti-C5 antibody BB5.1 (BB5.1) and its isotype control D1.3 hybridomas were also prepared in this way and then purified using an endotoxin-free method for use in the *in vivo* studies described in Chapter 4.

Hybridomas were cloned by limiting dilution in order to obtain a stable clone secreting high amounts of antibody. This clone was then inoculated at an initial density of 2 x 10^7 cells, suspended in 15mls low IgG R10 media into the lower compartment of a CELLine 1000 Integra flask (Integra Biosciences). This comprises a lower (small) and upper (large) compartment separated by a 10kDa semi-permeable membrane. 1L R+ media was then added to the upper compartment and the flask was incubated at 37°C, 5% $C0_2$.

Supernatant was harvested 1-2 times per week. This was done by first draining the upper compartment then during the initial period the cell suspension was removed from the lower compartment under sterile conditions and centrifuging at 450G for 5mins, supernatant was removed and the cell pellet re-suspended in 15mls of fresh low IgG media and replaced in the lower compartment of the Integra. Once a large pellet of cell had been achieved, 15mls of cell suspension was removed from the lower compartment and replaced with fresh low IgG media in order to maintain the cells at an optimal confluence and reduce the risk of contamination. The upper compartment was then refilled with 1L fresh R+ media and the Integra replaced at 37°C, 5% $C0_2$. Supernatant was centrifuged at 900G for 10mins x 2 in order to remove cell debris before being frozen at -20°C.

2.7 'Endotoxin-low' antibody purification

Hybridoma supernatant was thawed and pooled before high speed centrifugation at 1350G for 15 minutes at 4°C. Supernatant was then filtered through 0.22µM Stericup filter unit (Millipore) in order to remove any residual cell debris. All buffers were made in a class II cabinet in advance of purification. The AktaPrime protein purifier (GE Life Sciences) was pre-washed 3 times with 0.5M NaOH wash buffer to remove all traces of endotoxin. All lines were then rinsed x 3 with ultraclean water and checked with pH fix 0-14 litmus strips (Fisher) to ensure that all NaOH had been removed.

A 5ml Hi-Trap Protein G column (GE healthcare) was then attached to the Akta prime and Ab containing supernatant passed over the column. 0.1M Glycine elution buffer was used to elute protein from the column. Fractions were collected in sterile FACs tubes (BD biosciences) and neutralised using 100µl 1M Tris/HCl pH 9. Ab-containing fractions were identified using the absorbance trace via Primeview software. The column was cleaned between each cycle with ultraclean H_20 acidified to pH 1.5 with HCL.

Neutralised Ab fractions were pooled and loaded into Lyser dialysis cassettes (Fisher), dialysed three times into sterile PBS (Invitrogen) and then harvested into sterile tubes using a needle and syringe. Concentration was assessed using an A_{280} reading on the Nanodrop spectrophotometer (Labtech) before the antibody was frozen in aliquots in sterile freezing vials.

2.8 Preparing normal human serum

Normal human serum (NHS) was prepared from blood taken from healthy volunteers. This was collected into glass tubes and left at room temperature for 20mins with the edges of the forming clot being released intermittently. Following this, samples were then transferred to ice for a further 20mins. Samples were then

transferred to large falcon tubes and spun at 900 G for 10 minutes. The resulting supernatant and any loose cells were then transferred to a fresh falcon and spun for a further 10minutes at 900 G. Serum was then pooled, aliquoted and frozen at -80°C.

2.9 Complement Lysis Assay

K562 cells were incubated with titrated dilutions of supernatant from TG1 cells in Complement Fixation Diluent containing 0.1% gelatin w/v (CFD-g). Once coated in the TG1, K562 cells were exposed to dilutions of NHS (in CFD-g), as a source of complement. Ab and NHS doses were titrated in order to achieve 5-10% complement mediated cell lysis. The nucleic acid dye 7-amino-actinomycin D (7-AAD, BD pharminogen) was used to identify non-viable cells. 'Sub-lytically' attacked cells and controls were then coated with a titrated dose of TG1 such that surface levels were equivalent before being exposed to a lytic dose of NHS. Surface IgM was quantified using mouse anti-IgM biotin conjugated antibody followed by strepavidin, R-phycoerythrin conjugated antibody (both Invitrogen).
Each condition was performed in triplicate with assay volume tightly controlled and a fixed time and speed being used during sample acquisition. The level of cell lysis was calculated by counting the number of live cells at the end of the assay and expressing this as a percentage of the live cells present in the control (equivalent 1st step with no subsequent lytic step).

$$\frac{\text{(number of live cells in test condition)}}{\text{(number of live cells in internal control)}} \times 100 \qquad \text{(method A)}$$

2.10 CFSE labelling

K562 cells were washed in PBS then resuspended in 3ml warm PBS/0.1%BSA at a concentration of 2 x 10^6/ml. A 1uM carboxyfluorescein succinindyl ester (CFSE) (CellTrace, CFSE cell proliferation kit, (Invitrogen) solution was made using warm PBS/0.1%BSA. K562 were then added to CFSE (final concentration 0.5uM CFSE and

10^6 cell/ml). Following a 10 minute incubation at 37°C, cells were 'quenched' by adding 5 x volume of ice cold R10 then incubating on ice for 5mins. Following this, cells were centrifuged at 450 G for 5 mins, washed x 2 in CFD-g then resuspended at 1 x 10^6/ml and stored in the dark until use.

2.11 Ficoll Separation of PBMCs

Heparinised whole blood (200µl heparin/ 20 ml blood) was layered over an equal volume of Lymphoprep (Axis-Shield, Oslo, Norway) then centrifuged at 900 G for 20 mins at room temperature with no brake. A Pasteur pipette was then used to extract the PBMC layer from the Lymphoprep interface which was then washed twice in R10. 20µl of cell suspension was then mixed 1:1 with Trypan blue and counted using a haemocytometer under a light microscope.

2.12 NK Killing Assay

PBMCs were preconditioned overnight with or without 50IU/ml or 1000IU/ml IFNα (Roferon, Roche) in R10. The following day, K562 were labelled with CFSE, in order to discriminate them from PBMCs in co-culture. Labelled K562 were then exposed to sub-lytic complement. TG1 was re-titrated before cells entered a 4 hr NK killing assay using pre-conditioned PBMCs as a source of activated NK cells at a 5:2 effector: target ratio.

NK target lysis is conventionally expressed as:

$$\left(\frac{\text{dead targets}}{\text{total (live + dead) targets}} \right) \times 100 \qquad \text{(Method B)}$$

Due to target lysis and hence loss from the assay, Method A was reintroduced as a more accurate method of calculation.

(number of live cells in test condition) x 100 (Method A)

(number of live cells in internal control)

A modified experiment in which the sub-lytic step and NK kill occurred simultaneously was also developed in which the 10% FBS present in R10 was replaced with 10% normal human serum as a source of complement.

2.13 MACs removal of dead cells

A magnetic-activated cell sorting (MACS) based dead cell removal kit (Miltenyi Biotec) was used in accordance with the manufacturers' guidance following the initial sub-lytic attack in order to remove apoptotic and dead cells. Cells were re-suspended in 100µl of MACS bead, mixed and left at room temperature for 15 minutes. During this time binding buffer (BB) was prepared by diluting BB stock 1/10 in sterile, double distilled H_20. 500µl of BB was then used to prime each MACS column. Following incubation with MACS beads, 500µl of BB was added and the solution was then applied to the column. Columns were then rinsed 4 x with 500µl BB. Live cells present in the resulting effluent were washed twice in R10.

2.14 RNA extraction

A Qiagen RNeasy Mini Kit was used to extract RNA from the samples. All samples, which had been stored at 4˚C in RNAlater, were centrifuged at 450 G for 5 minutes to pellet the cells. Supernatant was then completely removed before cells were lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate-containing buffer which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol was then added to allow all RNA >200 nucleotides, to bind effectively to the membrane of the RNeasy mini column whilst washing away contaminants. The isolation of only RNA >200 nucleotides in length enriches for mRNA. RNA can then be eluted from the membrane using water.

The amount of RNA present in the eluted sample was then measured using a Nanodrop ND1000 (Labtech) spectrophotometer (Nanodrop). The Nanodrop was first used to set a baseline 'zero' absorbance reading at 260nm (A_{260nm}) using carrier only (water) and then sequential RNA samples were measured with the arm being cleaned and blanked with H_20 between each sample. An A_{280nm}/A_{260} ratio was calculated in order to assess purity and a desired ratio of ≈ 2 was achieved in all RNA samples.

2.15 Microarray

DNA microarrays simultaneously measure the expression levels of many genes by using probes specific to a DNA sequence within a given gene. The level of RNA hybridized to the probes is detected and quantified using fluorescent labelled target sequences allowing the gene expression level to be determined. Relative quantification is used to compare the intensity of a given probe in a sample exposed to a different condition. An Illumina Human HT-12v4 BeadChip, which uses probes immobilized to microscopic beads and analyses twelve samples simultaneously, was used.

2.16 Microarray statistical analysis

Microarray Analysis was completed in collaboration with the Bioinformatician, Dr Robert Andrews. The code an007.R was used to run the analysis and the lumi and limma packages in BioConductor were used to analyse the data. Raw data was inputed from GenomeStudio with replicate probes being averaged to a single value per probe. Data were normalised using Variance Stabilizing Transformation (VST) (the standard method for Illumina gene expression arrays) where data are background subtracted, VST transformed and quantile normalised.

Box plots of both raw and quantile-normalised data were used to assess the quality of the data. Hierarchical cluster analysis and principal component analysis were used to assess the natural clustering of the experimental groups. Hypothesis-driven

statistical analysis was then carried out in order to identify statistically significant changes in gene expression unique to the IP condition. Results were then validated by changing the normalisation method, building a batch effect into the model and removing potential outliers form the analysis. Methods and findings were reviewed by a second Bioinformatician.

2.17 Mice

Mice were bred and maintained in standard cages in the Joint Biological Services Unit (JBIOS), Cardiff University, Heath Park. Imaging studies were initially carried out in JBIOS before all imaging equipment was moved to a dedicated imaging suite, also on the Heath Park campus.

C3-/- mice were previously back-crossed onto C57BL/6 (B6) background at least 10 times (Morgan, Chamberlain-Banoub et al. 2006). C3aR-/- mice were originally obtained from Professor Craig Gerard and permission was granted to use these mice for these studies. C6-/- mice were available in our laboratory but had only been back crossed 7 times, these were therefore back-crossed onto B6 mice a further 3 times prior to commencing these studies. In experiments using knock-out mice, age-matched WT B6 mice aged between 6- 10 weeks were also used. All experiments were performed in compliance with Home Office regulations. Personal license number i49f49f29 (previously 30/9922). Project license 30/2891, protocol 2.

2.18 Splenocyte preparation

Individual spleens were gently crushed in 5ml PBS using the flat end of a 5ml syringe plunger through a 70μm cell strainer into a 50ml tube. A further 10ml of PBS was then added to the tube through the strainer. Cells were then centrifuged at 500 G for 5 min. Supernatant was removed and the cell pellet re-suspended in 5ml red cell lysis buffer diluted 1/10 with sterile water. After 90 seconds, 15mls of PBS was added and cells were centrifuged for 5 mins at 500 G. Cells were then re-suspended

in 5mls R10, removing any visible cell debris. Cells were counted and resuspended in facs buffer or R10 at the desired concentration.

2.19 Murine serum collection

Mouse serum was either harvested from live mice following tail tipping and milking of 100µl into a collection vial or following schedule 1 sacrifice when blood was collected by intrathoracic cardiac aspiration. Blood was clotted on ice for 15 minutes before being spun at 450 G for 10 minutes at 4°C before the upper serum fraction was used immediately or stored at -80°C.

2.20 C1498 cell inoculation

Cells were thawed 4- 7 days prior to each experiment. Cell viability and density was assessed and cells passaged on day 1 post thaw and the day prior to inoculation. Viability was assessed using cells pre-mixed with 1/1 Trypan blue solution (Fluka) and was required to be >95% in order for tumour cell inoculation to proceed. Cells were counted using a haemocytometer and resuspended at 1 or 5 x 10^7 cells/ ml in sterile PBS such that 100µl would provide 1 or 5 x 10^6 cells as per experimental protocol.

For intravenous (IV) studies inoculums of 10^6 and 5 x 10^6 were explore in my initial experiment with animals in all subsequent experiments receiving 5 x 10^6 C1498 cells IV. These were administered via tail vein injection into study animals which had been warmed for >15mins prior to injection to achieve maximal vasodilation. For subcutaneous (SC) studies inoculums of 10^6 were injected into the left lateral abdominal wall.

2.21 *In vivo* imaging

Experimental mice were first anaesthetised with inhaled isofluorane which was maintained throughout the imaging process. The abdomens of mice were shaved to

allow optimal imaging. Kodak FX Pro fluorescence imaging equipment (FX-Pro) was used to image DSRed positive leukaemic infiltrations using an excitation filter of 550nm and emission filter of 600nm in conjunction with an exposure time of 20 seconds. Fluorescent images were superimposed on white images to allow accurate disease localisation of the cellular DSRed fluorophore in malignant tissue. A combined imaging and health (IH) score was devised which scored both imaging findings and health factors to allow consistent decisions to be made on experimental endpoints (Table 2.2).

Mouse Health	**Points**
Minor behavioural- easier to catch, not trying to escape	1
Piloerect, dishevelled	2
Palpable lump	3
Abnormal posture or locomotion	4
Definite symptoms of ill health- piloerect, hunched, isolated	4
Imaging finding	
Lesion visible when settings adjusted but not at experimental settings	1
Single positive lesions	2
Multiple positive lesions	3
Full body bioluminescence	4

Table 2.2: Imaging and Health (IH) Score. This incorporates both imaging findings and health factors in a combined score allowing consistent decisions to be made on experimental endpoints. Initial experiments used an IH score of 4 points as the experimental endpoint. This was later revised to 3 and then 2, in order to minimise any ill effects experienced by the mice.

Following euthanasia, livers, spleens and ovarian tumours were harvested and preserved in optimal cutting temperature compound (OCT) at -80°C or in 4% paraformaldehyde solution (PFA) at RT prior to being embedded in paraffin.

2.22 T and NK cell depletion

The anti-NK antibody PK136 was used at a dose of 0.5mg IP (2 mice). Two anti-CD8 antibodies (YTS-169 and YTS-156) were used in combination, each at a dose of 100µg IP (2 mice). Dosing was repeated 3 days later. On day five these mice along with a control mouse were harvested and their spleens analysed for the presence of NK and CD8+ T cells. Splenocytes from mice treated with NK or CD8 depleting antibodies along with an untreated control were first stained with live/dead aqua then with pacific blue conjugated anti-CD8a, Fitc conjugated anti-NK1.1 or isotype controls antibodies as listed in Table 2.1.

For *in vivo* depletion studies groups of eight 7-13 week old C3-/- or age matched WT mice were injected on day -3, day -1 and then fortnightly with a total of 200µg of either CD8 depleting (100µg of each YTS-169+ YTS-156), CD4 depleting (100 µg of each YTS-191 and YTA-3) or control (GL113) antibodies.

2.23 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

In order to determine whether the C1498 cell line was producing C3, both cell lysate and 20 x concentrated cell supernatant were analysed by SDS-PAGE. Based on preliminary studies a 1/25 dilution of mouse serum and a 1/50 dilution of purified mouse C3 were used as positive controls with undiluted C3-/- mouse serum being used as a negative control. C1498 supernatant was 20 x concentrated using an Amicon pressure ultrafiltration system. A C1498 cell lysate was produced by first washing cells x 3 in PBS before snap freezing the cell pellet in liquid nitrogen. 20µl of each protein containing sample was then boiled for 2-5 minutes with 10µl of loading buffer under reducing conditions. A ready-made NuPAGE SDS PAGE 4-12% Bis-Tris gel was submerged in 1 x NUPAGE MES SDS-PAGE running buffer (both Life Technologies). Duplicates of 15µl of protein solution were loaded into wells along with pre-stained broad range markers in order to allow size estimation (New England Biolabs). The gel was subjected to 200V for 60 minutes.

2.24 Western blot

Resolved gels were submerged in TRIS glycine transfer buffer and blotted onto nitrocellulose membrane sandwiched between filter paper and sponges pre-soaked in transfer buffer and placed in a cassette at 30V for 60 minutes. Following transfer the nitrocellulose membrane was removed and blocked with 5% PBS-milk for 30 minutes at room temperature. After washing in PBS T the membrane was incubated with HRP conjugated goat anti-mouse anti-C3 mAb at a 1/10,000 dilution in 0.5% PBS-milk for 60 minutes. The membrane was then washed with PBS-T before bound antibody was visualised using an enhanced chemiluminescence kit (GE Healthcare), subjected to light sensitive hyperfilm (GE Healthcare) and developed using a Compact X-2 X- Ograph developer machine.

2.25 Immunohistochemistry

Fresh tumour samples were immediately embedded in OCT compound and frozen on dry ice before being stored at -80°C in freezers until use. 5µm thick sections were cut and placed on slides. Sections were fixed in ice cold acetone for 15mins then washed x 3 in PBS. Sections were encircled using a PAP pen then blocked with Donkey serum in PBS/2% BSA 200µl per section for 40 mins at RT. After washing with PBS, samples were incubated with either an anti-mouse C3 antibody (11H9) or a rat IgG2a isotype control, at a final concentration of 2µg/ml overnight in a humidity box at 4°C. The following day samples were washed x 3 in PBS then incubated for 30 mins at RT in 488 anti rat secondary antibody at 1:500. Following 3 x PBS washes, samples were fixed for 10 minutes in 1% PFA then washed a further 3 x in PBS. DAPI stain (vector shield) was then applied to a cover slip onto which the slides were placed. The slides were then sealed with nail polish prior to analysis.

2.26 Vaccination

5 x 10^7 irradiated (10,000 rad) C1498 cells were injected SC into WT and C3-/- mice. This was repeated after 4-6 weeks. 7 days after the second vaccination, vaccinated

mice along with unvaccinated WT and C3-/- controls were euthanized and their splenocytes used in ELISpot assays.

2.27 IFN-γ ELISpot Assays

Polymer-backed 96-well filtration plates (MAIP-S-4510) (Millipore, Moslheim, France) were used for ELISpot assays along with an IFN-γ ELISpot ALP Kit (Mabtech 3321-2A). The plate was first pre-wetted for 2 mins with 70% ethanol, 50µl/ well then washed 5 x with sterile PBS. Wells were then coated with 50µl of 15µg/ml rat anti-mouse anti-IFN-gamma capture antibody (1-DIK). Plates were tapped gently to ensuring the whole well was covered before being incubated overnight at 4°C. The following day, wells were washed x 5 with PBS to remove excess antibody then blocked with 50µl R10 for > 1 hour at 37°C.

Effectors, splenocytes and targets, irradiated C1498, were each seeded in a volume of 50µl in R10 at a range of target: effector ratios. 50µl of phytohaemogglutinin (PHA) at a final concentration of 10µg/ml was used as a positive control with 50µl R10 being used in negative controls such that the final volume of each well was 150µl. All conditions were performed in triplicates. Plates were then incubated at 37°C for 18-24hrs. The next day cells were removed and plates washed x 6 with PBS. 50µl of 1µg/ml biotinylated rat anti-mouse interferon-gamma (7-B6-1) was added to each well and incubated for 2 hours at room temperature. Plates were then washed x 6 in PBS before adding 50µl Streptavidin-Alkaline Phosphatase Polymer (Mabtech, Sweden) diluted to 1µg/ml in PBS and incubated at room temperature for 1 hour. Following 6 further washes, the ELISpot wells were developed using 50µl/well of colour development buffer (Biorad AP Conjugate sub kit 170-6432, Hercules, California) and incubated at room temperature until spots were clearly visible (approx 10-15mins). The reaction was then stopped by washing plates in tap water and left to dry overnight. IFN-γ- producing T cells were enumerated at the single-cell level by counting the number of spots per well using an automated ELISpot plate reader (Autoimmun Diagnostika GMBH, AID, Strasberg, Germany) and

analysed using the ELISpot 5.0 software package to ensure consistent analysis of spots between wells before manually verifying spot counts.

2.28 Validating Efficacy of the anti-C5 antibody BB5.1

BB5.1 and its isotype control D1.3 were produced and purified using methods described above. Following purification, the efficacy of BB5.1 vs D1.3 was assessed using an *in vitro* haemolysis assay using sensitized rabbit erythrocytes. The *in vivo* efficacy of BB5.1 following a single dose of 1mg IP vs isotype control was assessed using complement haemolysis assays on blood taken prior to and at 3 time-points following administration.

2.28.1 Haemolysis assays

Rabbit erythrocytes were first coated with antibody in order to sensitize them for complement attack. This was done using rabbit blood that had been mixed with an equal volume of Alsever's following harvest, in order to preserve cells and prevent clotting. Cells were centrifuged at 450 G for 5 minutes at 4°C then diluted in 4% CFD. Polyclonal mouse anti-rabbit erythrocyte antibody was diluted to 0.5% v/v in CFD then incubated 1:1 with the rabbit erythrocyte suspension for 30 minutes at 37°C. Cells were then washed (450 G, 5 mins, 37°C) in order to remove any unbound antibody before being re-suspended in 2% CFD.

Duplicate wells of 50µl mouse serum were plated with 50µl of CFD into a 96-well round bottomed plate and then ½ serially diluted down. Duplicate positive controls containing 50µl dH_20 and a negative control containing 50µl CFD were also included. 50µl of antibody coated rabbit erythrocytes were added to both test and control wells and incubated for 30 minutes at 37°C. Intact erythrocytes were pelleted by centrifugation (450 G, 5 mins). 50µl of erythrocyte supernatant from each test and control well was then added to a flat-bottomed plate containing 100µl dH_20 per well. Absorbance of each well was read at 415nm using a Dynex MRX II plate reader.

Percentage lysis was calculated using the positive control to provide 100% lysis and the negative 0% lysis using the following equation:

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

A 1/25 dilution of serum resulted in 60% lysis and was taken forward to assess the ability of the anti-C5 mAb vs control Ab to deplete C5 *in vitro.* Abs were diluted to 200µg/ml in CFD. 50µl was plated in duplicate and ½ serially diluted in CFD. 1/25 mouse serum and Ab coated erythrocytes were then added resulting in a final top concentration of 50µg/ml. The level of haemolysis was then measured as described above.

2.29 *In vivo* complement depletion

A dose of 1mg BB5.1 was administered via an IP injection twice weekly beginning on day -1 (day 0 being the day of tumour cell injection). An isotype control antibody (D1.3) was used at an equivalent dose in age- and sex-matched control mice.

The Cyclic hexapeptide antagonist AcF-[OPdChaWR], also known as PMX-53 specifically blocks the main C5a receptor (Markiewski, DeAngelis et al. 2008; Corrales, Ajona et al. 2012; Vadrevu, Chintala et al. 2014) and was kindly provided by Professor Trent Woodruff. C5a receptor antagonist (C5aRA) was used at the dose described in other studies (1mg per kg) in both a 3 times per week and a daily dosing regimen. Treatment commenced the day prior to administration of C1498 cells and continued for the duration of the experiment. Mice were imaged twice weekly from day 7.

An antisense oligonucleotide which targets C6 mRNA preventing the synthesis of C6 in the liver and hence the production of MAC was used to reduce C6 synthesis in WT mice. The C6 antisense along with a control oligonucleotide was kindly provided by Professor Kees Fluiter (Fluiter, Opperhuizen et al. 2014). A dose of 5mg/kg dose

subcutaneously (SC) three times a week was used, in line with his recommendation. This was commenced the day prior to administration of 5 x 10^6 C1498 cells IV.

2.30 Statistical and Graphical Analysis

Analysis of flow cytometry data was performed using FlowJo version 7.6.5. Statistical analysis of my *in vivo* studies was carried out using Graphpad Prism version 5. Kaplan Meier survival curves were used to plot results with log-rank (Mantel-Cox) tests being used to assess statistical significance between experimental groups.

Chapter 3: An exploration of whether complement-induced protection is protective against natural killer cell attack *in vitro* and whether 'protected' cells have a genetic signature

3.1 Introduction

As described in Chapter 1, sub-lytic doses of complement protect cells from a subsequent lytic dose of complement, a phenomenon termed complement-induced protection (CIP) (Reiter, Ciobotariu et al. 1992). Cross-protection is also observed between other pore forming proteins, such as perforin, melittin and streptolysin, that are incorporated into cell membranes via a similar mechanism (Reiter, Ciobotariu et al. 1995). This is important to these studies as NK cells utilise perforin in order to form the pores through which inducers of cell death, such as granzymes, are introduced to the target cell. It is therefore possible that sub-lytic complement protects tumour cells from lysis by cytotoxic cells, including NK cells and T cells.

The myeloid leukaemia cell line K562 was the original cell line in which CIP was described (Reiter, Ciobotariu et al. 1992). These studies utilised polyclonal anti-K562 antibodies and pooled normal human serum (NHS) in order to create both sub-lytic and lytic complement attacks with chromium-release assays being used to determine levels of cell death (Reiter, Ciobotariu et al. 1992). The aims of experiments described in this Chapter were to develop a flow cytometric method to demonstrate CIP *in vitro,* to use this assay to determine if exposure to sub-lytic complement also protects cells from lysis by natural killer (NK) cells *in vitro* and finally, to determine a genetic signature for CIP.

In order to develop a flow cytometric method capable of demonstrating CIP *in vitro* the K562 cell line was also used. TG1, an IgM anti-myeloid antibody (Ab) was used to coat the K562 cells in order to target them for complement-mediated lysis with NHS (see Chapter 2 for details).

In order to convincingly demonstrate the phenomenon, it was important to ensure that an appropriate level of sub-lytic attack had occurred in each experiment. It was also essential that an equivalent level of target Ab was present in all test conditions prior to the 2nd lytic complement attack.

Once developed the flow cytometric assay was altered in order to substitute the second lytic complement attack for a natural killer (NK) cell killing assay. Donor PBMCs were conditioned overnight with interferon α (IFNα) and then used as a source of NKs in the killing assays (see Chapter 2 for details). The effect of Ab, NHS, donor, effector:target (E:T) ratio, time-scale and alternative experimental methods were assessed in order to explore whether exposure to sub-lytic complement protects cells from lysis by NK cells *in vitro*.

The third part of this chapter investigates whether CIP is the result of altered gene transcription. CIP has been shown to be dependent on both protein and RNA synthesis. The use of protein synthesis inhibitors results in complete loss of the protective phenotype whilst RNA synthesis inhibitors led to a partial loss of phenotype (Reiter, Ciobotariu et al. 1992). It was therefore reasonable to assume that a sub-lytic complement attack which resulted in a protected phenotype would lead to a specific genetic signature.

This was investigated by performing a microarray transcriptome analysis on RNA extracted from cells that had been exposed to sub-lytic complement and confirmed to have a protected phenotype. This section aimed to define the genetic signature of CIP.

3.2 Results

3.2.1 Develop a flow cytometric method of demonstrating complement-induced protection *in vitro*

3.2.1.1 Establishing a sub-lytic level of complement attack

Conditions required for a sub-lytic level of complement attack were established by examining cell lysis following incubation with titrated doses of TG1 Ab and NHS. The aim was to identify a level of cell death which was 5-10% above control cells, which had not been exposed to Ab. This level of cell death was a surrogate marker that cells which survived the complement attack had been subjected to a sub-lytic dose of MAC. This was achieved by keeping NHS dose constant at 1/8 dilution and diluting TG1 Ab (Figure 3.1).

3.2.1.2 Re-titrating TG1 prior to lytic complement attack

Following sub-lytic attack, cells were re-exposed to TG1 prior to the second complement attack. It was shown that cells previously exposed to TG1 could not be saturated with Ab following a second exposure, probably due to antigen capping (Figure 3.2). This was an important finding as different levels of TG1 binding would affect the extent of lysis observed during the second kill step and could therefore be misleading. It was therefore essential that controls were coated with titrated levels of Ab prior to the lytic complement step such that surface Ab levels (as measured by an anti IgM Ab) were comparable to 'protected' cells prior to exposure to lytic doses of NHS (Figure 3.3). In each case, a control with a slightly lower level of surface TG1 was chosen as a comparator such that any bias was in the favour of the control.

3.2.1.3 Effect of Sub-Lytic MAC on Lytic Complement Attack

Following the sub-lytic step and re-exposure to titrated TG1, cells were exposed to a range of lytic doses of complement. Cell death was assessed using 7AAD staining.

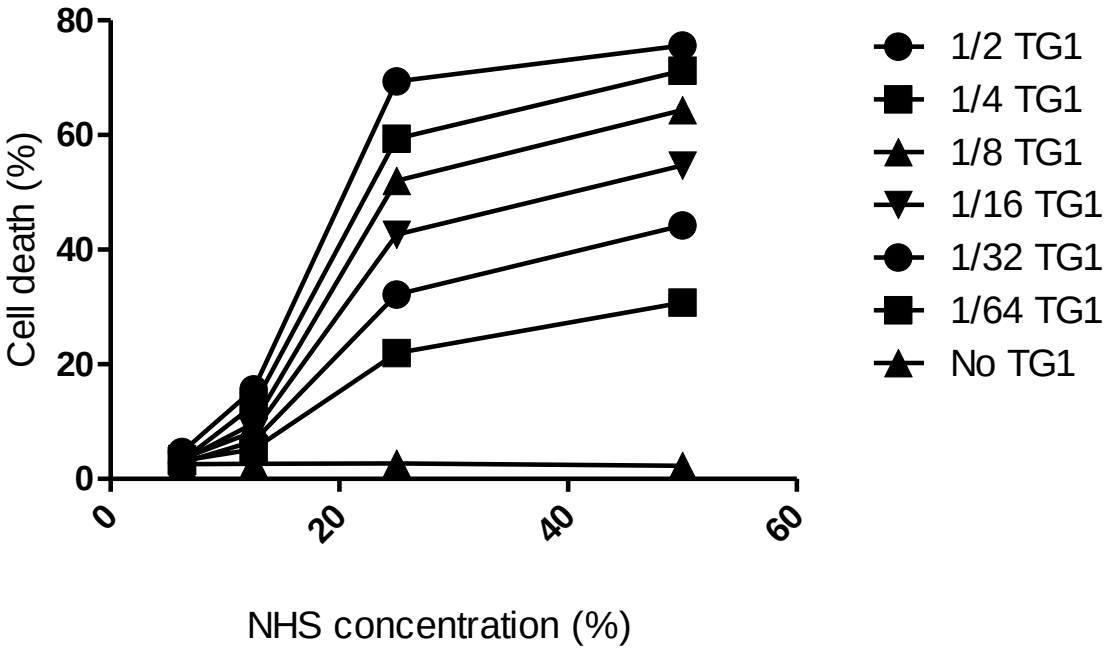


Figure 3.1: TG1/ NHS killing curve. K562 cells were first incubated with titrated dilutions of supernatant from anti-myeloid cell IgM mAb producing TG1 cells in CFD containing 0.1% gelatine (CFD-g). These cells were then exposed to a range of dilutions of NHS, as a source of complement (also diluted in CFD-g). 7AAD staining was used to identify non-viable cells. TG1 and NHS doses were titrated in order to achieve 5-10% complement-mediated cell lysis.

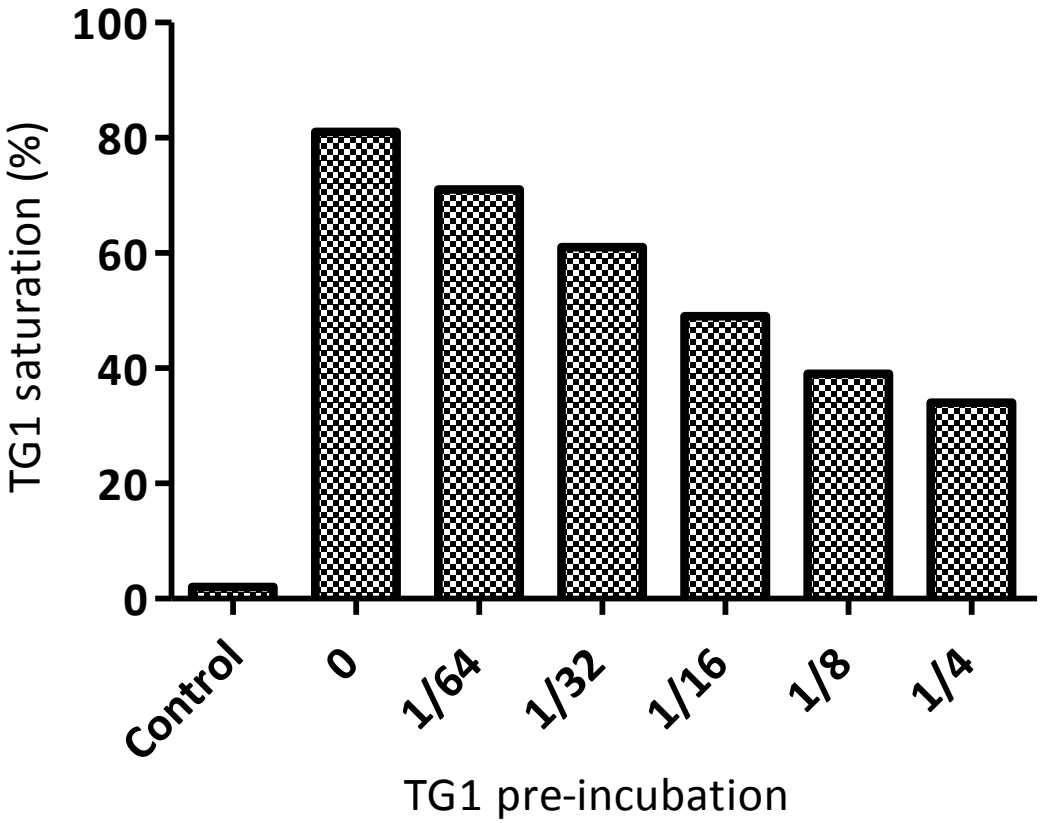


Figure 3.2: TG1 saturation post second incubation. K562 cells were first exposed to a variable dilution of TG1 then to a ½ dilution of TG1. TG1 saturation following the second exposure is plotted. This demonstrates an inability to saturate cells that had had a prior exposure to TG1, in a dose dependent manner.

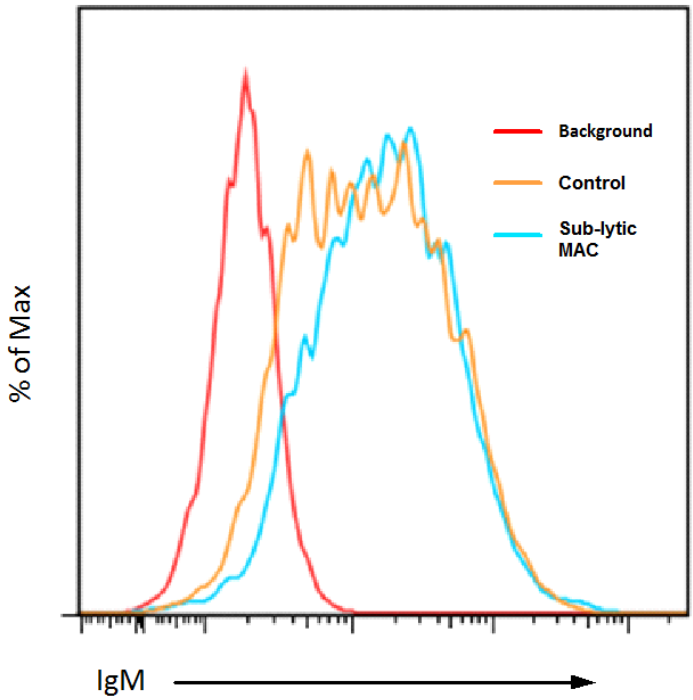


Figure 3.3: TG1 saturation prior to second lytic complement attack. A representative plot showing levels of surface IgM (TG1) following a 2nd incubation with the TG1 Ab containing supernatant, prior to the 2nd 'lytic' step. Levels of surface IgM were assessed using a biotynylated anti-IgM Ab conjugated to strepavidin PE. In each case, a control with a slightly lower level of surface TG1 was chosen as a comparator such that any bias was in the favour of the control.

Percent survival was measured using a calculation based on live cell numbers present at the end of the experiment.

$$\frac{\text{(number of live cells in test condition)}}{\text{(number of live cells in internal control)}} \times 100 \qquad \text{(Method A)}$$

Tightly controlled assay volume, time and speed of sample accrual and triple repeats were all used to ensure accuracy. A schematic demonstrating the experimental protocol is shown in Figure 3.4.

This flow-cytometric assay confirmed that sub-lytic complement attack offered protection against a subsequent lytic complement attack (Figures 3.5 and 3.6).

3.2.2 Determine if exposure to sub-lytic complement protects cells from lysis by NKs cell *in vitro*

3.2.2.1 Experimental protocol

The experimental protocol described above was modified in order to determine whether a sub-lytic dose of complement was also protective against NK cell attack. Both NK and $CD8^+$ cytotoxic T lymphocytes utilise perforin in order to insert pores and lyse cells via a similar mechanism to MAC. A NK killing assay, using normal donor peripheral blood mononuclear cells (PBMCs) pre-conditioned overnight with variable doses of IFNα as a source of NK cells, was adapted from the above protocol (Figure 3.7).

CFSE was used to label target K562 cells such that they could be discriminated from the effector PBMC cells. Dual staining with 7AAD allowed the level of cell death to be measured, allowing an accurate calculation of the level of target cell lysis. By applying the same gating strategy to a PBMC-only sample I confirmed that dual staining led to no overlap between targets and effector cells (Figure 3.8).

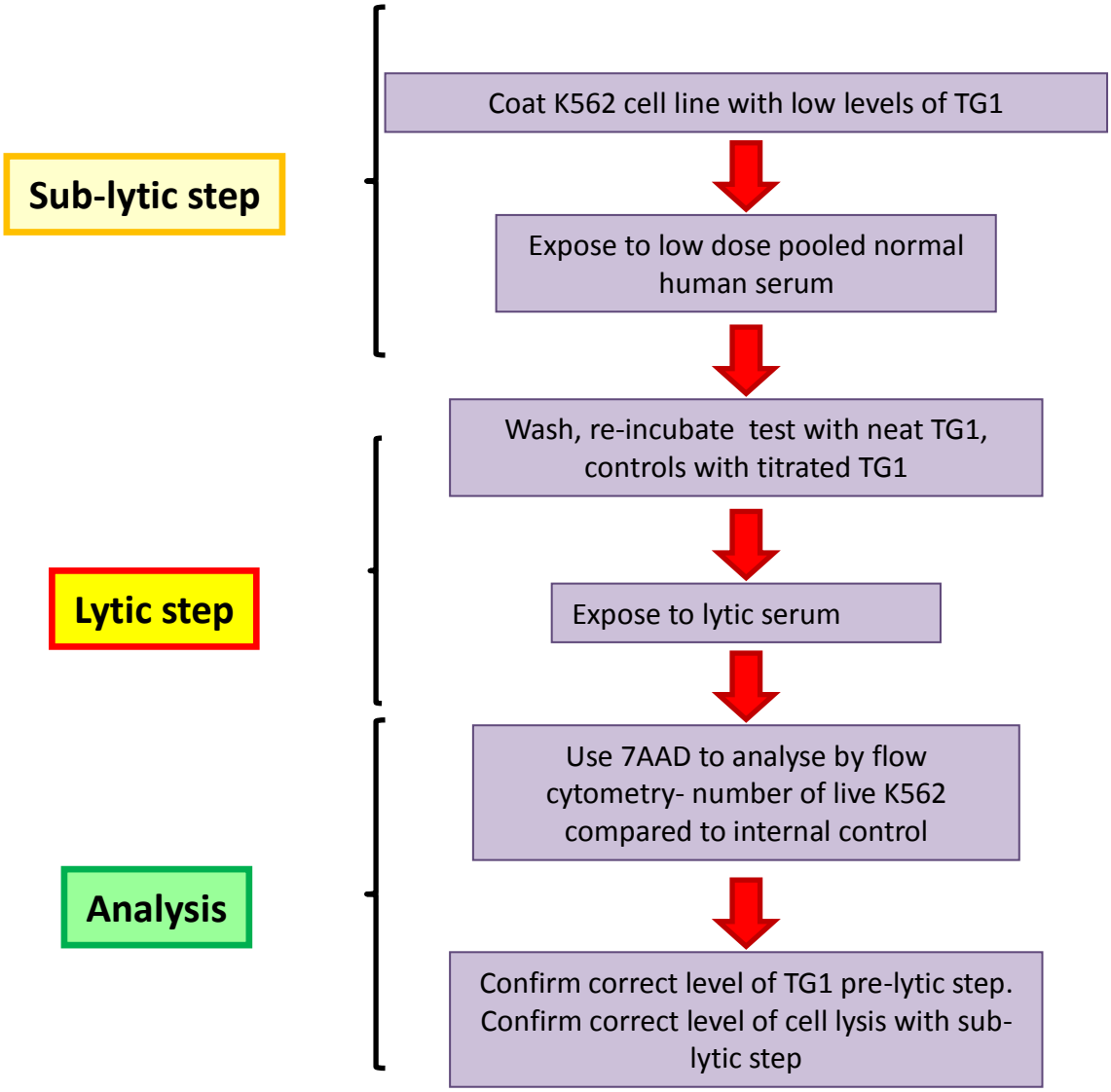


Figure 3.4: Schematic of fluorescent-based flow cytometric assay developed to demonstrate complement-induced protection. TG1 was used to sensitise K562 to complement attack using NHS as a source of complement. Following a sublytic attack, defined as 5-10% cell death, the surviving cells were exposed to a lytic complement attack.

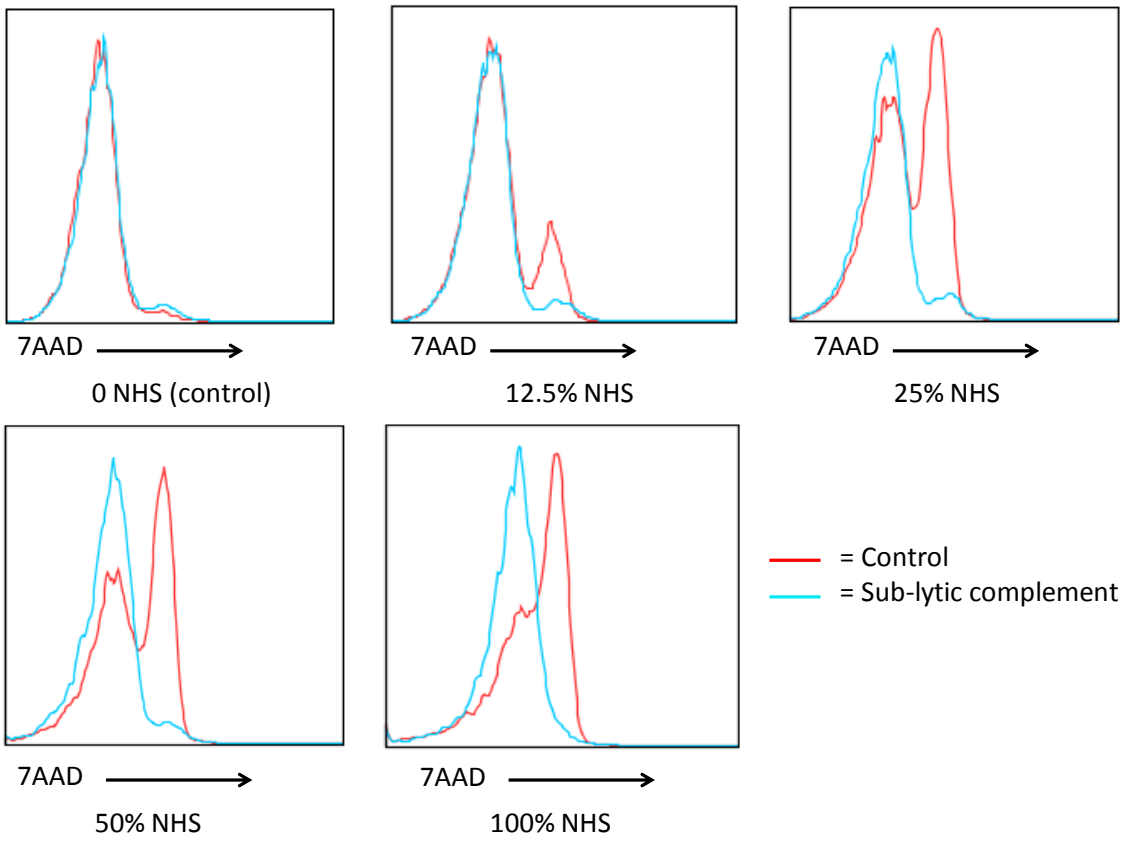


Figure 3.5: Flow cytometric plots demonstrating complement induced protection. Following a sub-lytic complement attack and re-titration of antibody on their surface, K562 cells were exposed to variable dilutions of NHS as a source of complement. 7AAD was then used to assess cell viability. In this example, the absence of a 7AAD +ve peak in the 'protected' (blue) vs control (red) cells reflects almost complete protection at all NHS concentrations.

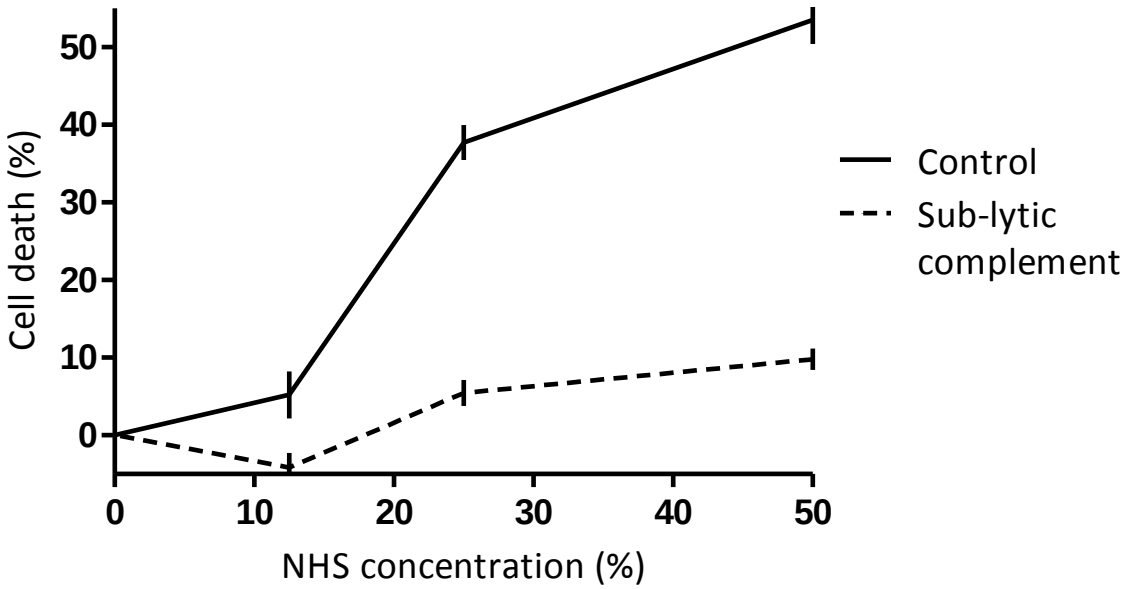


Figure 3.6: Killing curves demonstrating complement-induced protection. Cells exposed to sub-lytic MAC (1/4 TG1+ 1/8 NHS) were protected from a range of lytic doses of complement when compared to control cells.

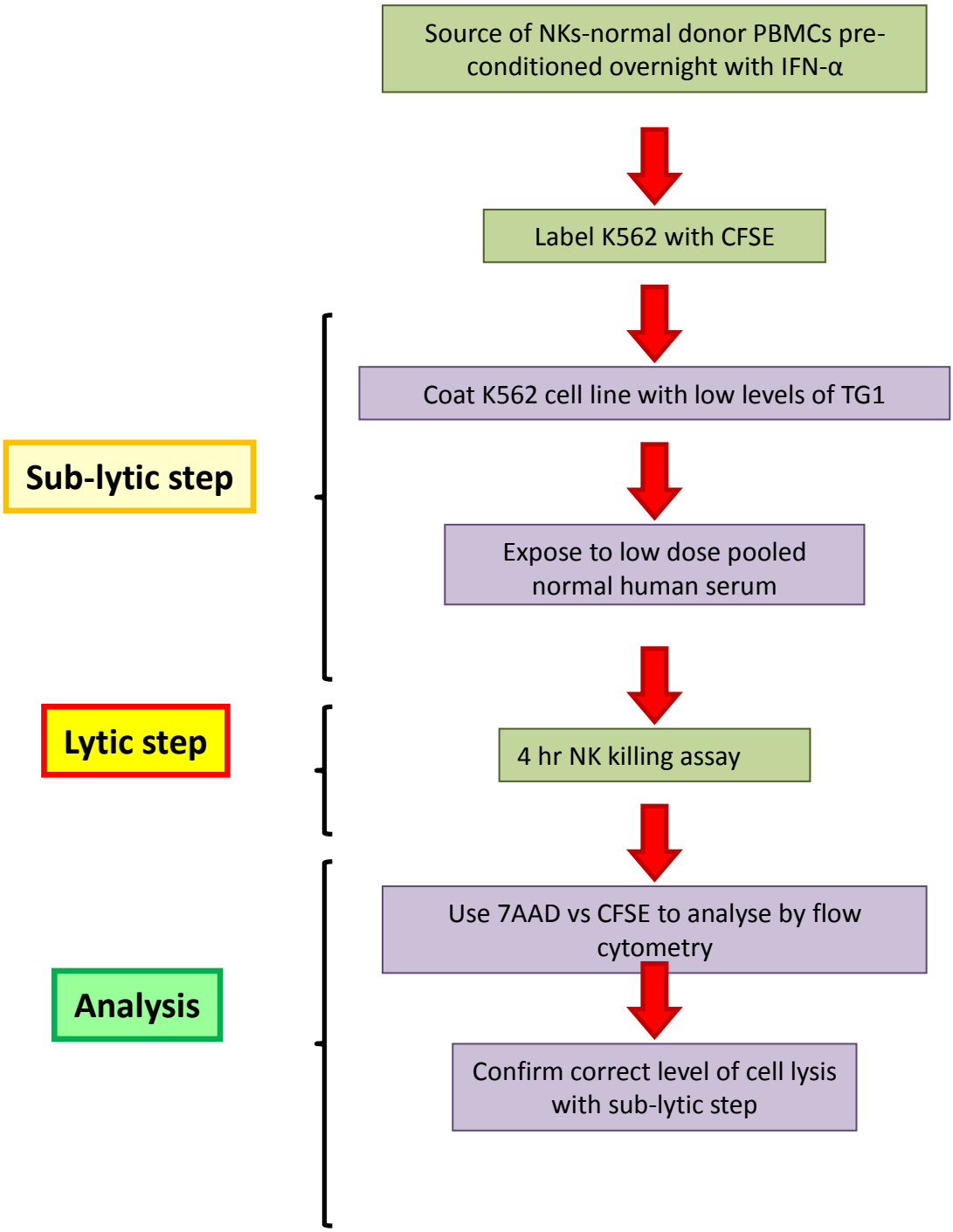


Figure 3.7: Schematic showing modifications used to assess complement-induced protection against NK cell cytotoxicity. An NK killing assay was incorporated into the original assay, using IFN-α conditioned PBMCs as a source of NKs. K562 target cells were labelled with CFSE to enable them to be distinguished from effectors and allow killing to be quantified. K562 cells which had been exposed to a sub-lytic dose of complement then entered a 4 hr NK killing assay. At the end of this time, 7AAD and CFSE staining were used to calculate cell death

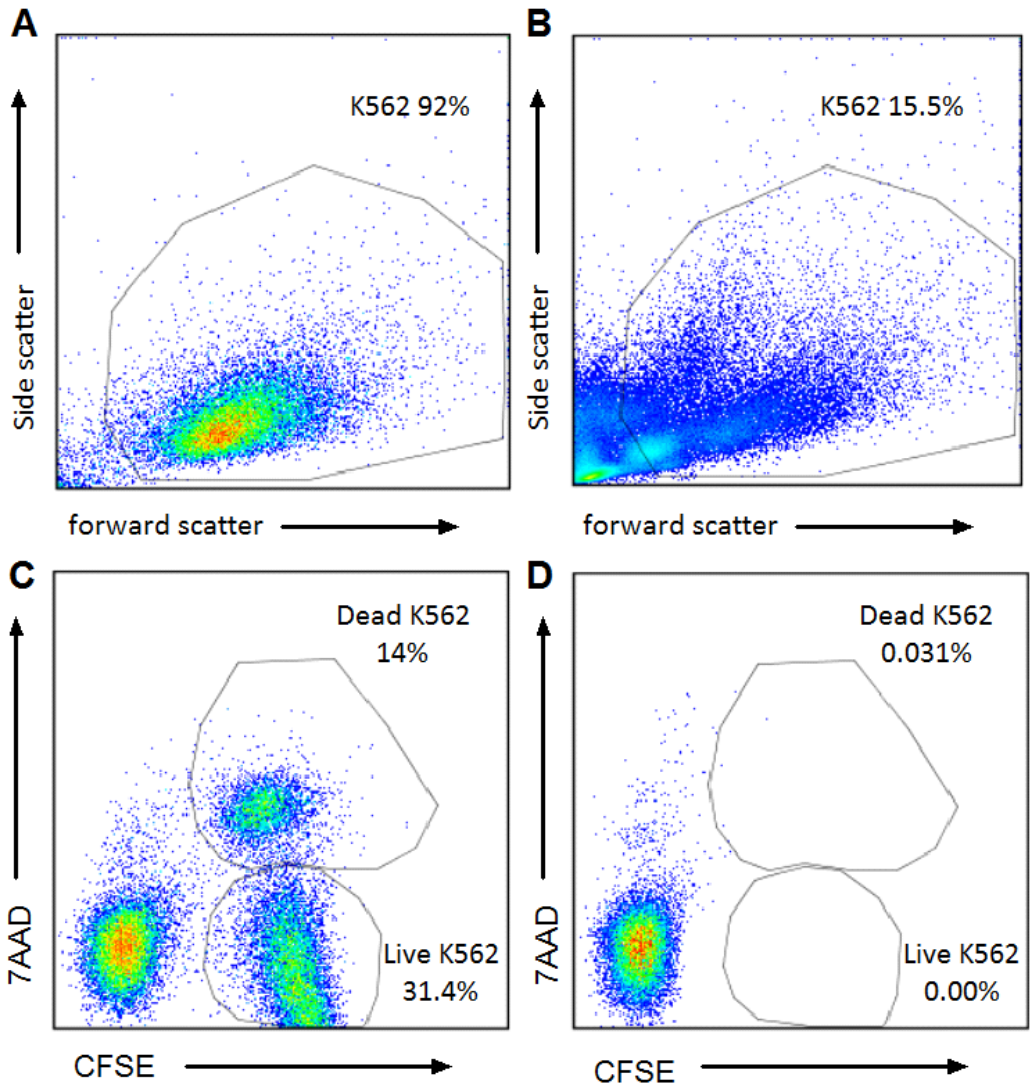


Figure 3.8: Gating strategy used in NK assays. Cells were first gated on forward vs side scatter A) K562/ targets only, B) full killing assay (K562 + PBMCs). Assays were then gated on CFSE vs 7AAD, the CFSE staining allowing the +ve target K562s to be distinguished from the –ve effector PBMC. 7AAD staining identified whether a cell was live (-ve) or dead (+ve). C) this gating strategy allowed calculation of the level of target lysis. D) analysis of a sample containing PBMCs only confirmed no overlap into live K562 gate from PBMCs.

3.2.2.2 Effect of Antibody and Serum on NK Killing

The presence of TG1 Ab on the target cell surface was assessed in order to see whether this would have an impact on NK killing activity. Cells exposed to variable concentrations of TG1 Ab were compared with controls, demonstrating that increasing amounts of the Ab did appear to lead to a slightly higher rate of target cell lysis (Figure 3.9). It was therefore concluded that it was critical to titrate and control for the level of TG1 Ab prior to the NK killing assay, as with the complement assays described above.

The presence of NHS as opposed to foetal bovine serum was next assessed in order to determine whether this had an impact on the level of NK mediated cell lysis. The source of serum was shown to have no impact on NK mediated cell lysis (Figure 3.10).

3.2.2.3 Method of Calculating NK Mediated Cell Lysis

NK killing is conventionally calculated by expressing dead/ 7AAD positive cells as a percentage of total targets in the assay.

$$\left(\frac{\text{dead targets}}{\text{total (live + dead) targets}} \right) \times 100 \qquad \text{(Method B)}$$

Initial experiments, using this method appeared to demonstrate that sub-lytic complement attack did indeed lead to a degree of protection from subsequent NK cell lysis (Figure 3.11).

However, it was noted that in the setting of a prior complement attack, the conventional method of NK analysis appeared to be inaccurate. This was due to cells being completely lysed and lost into the debris gate and therefore not being counted in the dead K562 gate. Figure 3.12 demonstrates the increased incidence of 7AAD+ve debris in the samples that had been exposed to sub-lytic complement as

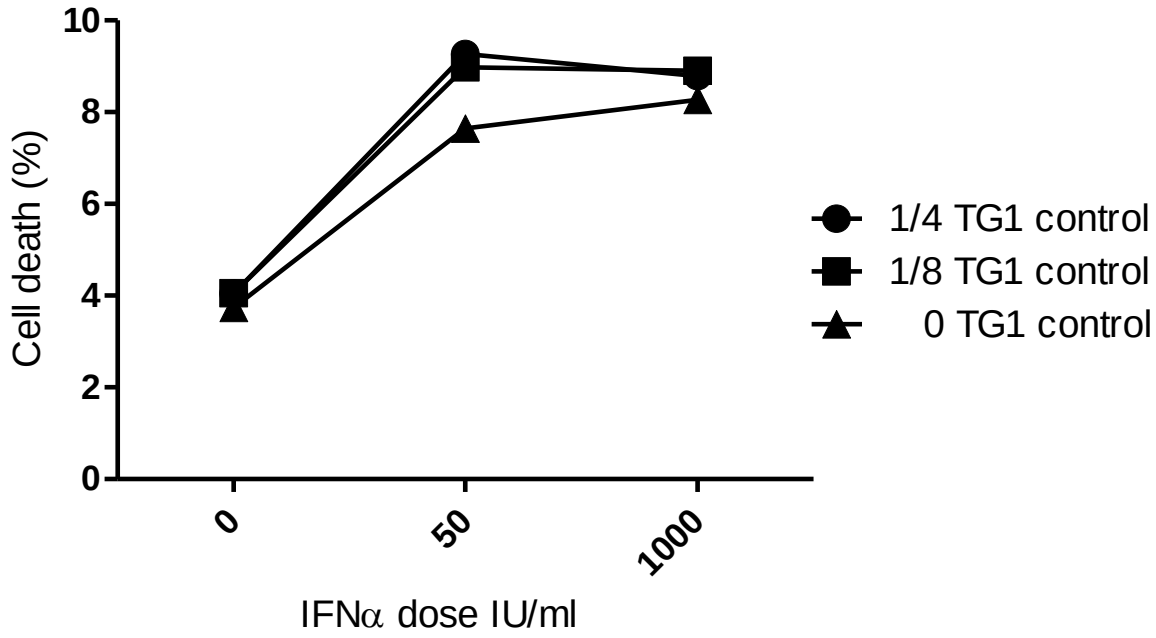


Figure 3.9: Effect of TG1 on NK mediated cell death. PBMCs pre-conditioned overnight with 0, 50 or 1000 IU/ml of α interferon (IFNα) were used as effectors in a NK killing assay against K562 cells which had or hadn't been coated with TG1 Ab at a E:T ratio of 5:2. A reproducible slightly higher NK mediated cell death was observed when K562 cells were coated with TG1 anti-myeloid cell Ab compared to untreated controls.

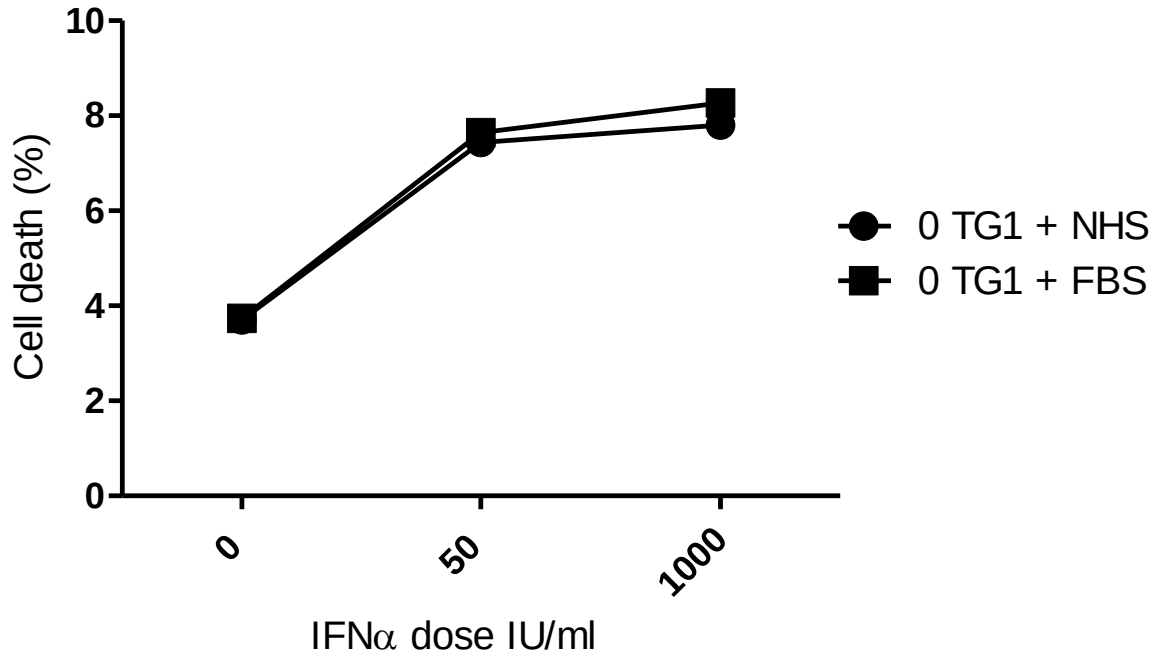


Figure 3.10: Effect of normal human serum on NK mediated cell death. PBMCs pre-conditioned overnight with 0, 50 or 1000IU IFNα were used as effectors in a NK killing assay against K562 cells in the presence of either 10% NHS or 10% fetal bovine serum (FBS). The presence of NHS vs FBS had no effect on the level of NK mediated cell death.

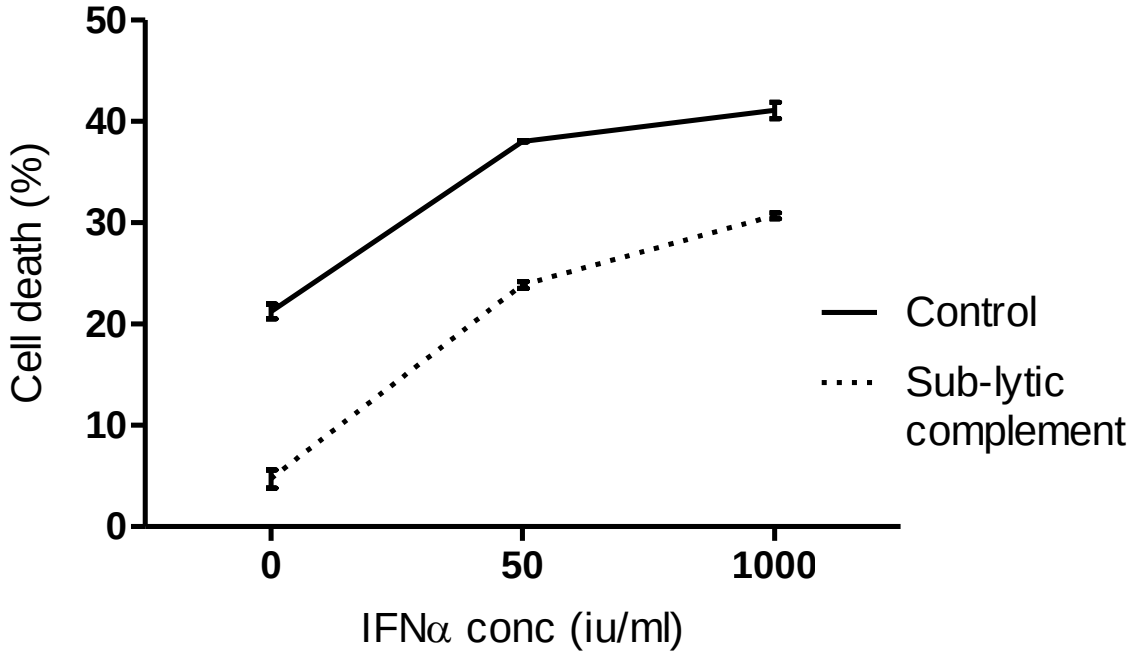


Figure 3.11: Initial NK assays using conventional methods of calculating cell lysis. PBMCs pre-conditioned overnight with 0, 50 or 1000 IU/ml IFNα were used as effectors in a NK killing assay against K562 cells. Results were calculated using 7AAD positive cells as a percentage of total targets in the assay. This appeared to show that cells exposed to sub-lytic complement (1/16 TG1 + 1/8 NHS) were protected from subsequent NK attack.

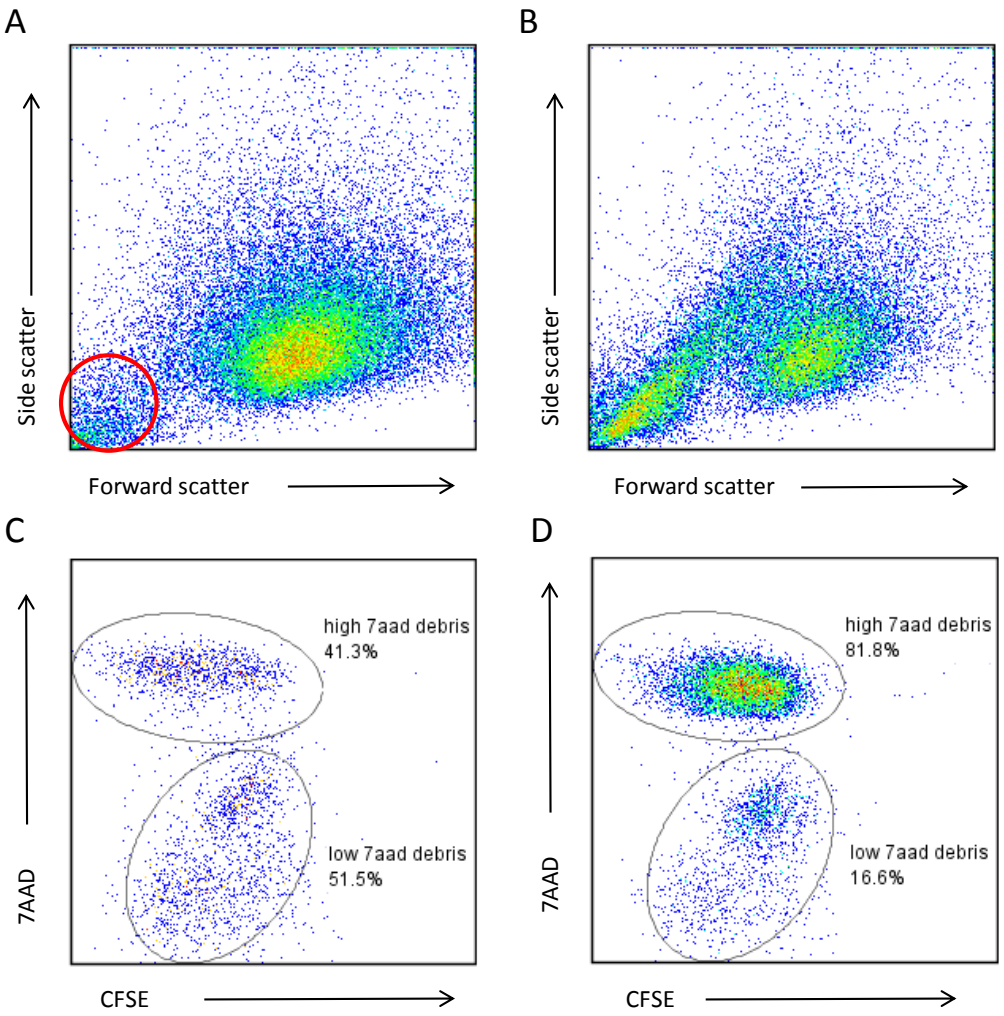


Figure 3.12: Flow cytometric plots demonstrating increased incidence of 7AAD positive 'debris'. Control samples (A) had less events recorded in the area where cell debris usually occurs. Cells which had previously been exposed to 'sub-lytic' complement attack (B) had far more cells in the 'debris' gate. 41% of events recorded in the debris gate of control samples were 7AAD positive (C). Debris from samples that had undergone a sub-lytic complement attack had almost twice the level of 7AAD positive debris (81.8%) (D). This suggests that these were lysed dead cells.

compared to controls. The higher numbers of cells lost from conditions that had previously undergone sub-lytic attack appeared to equate to the level of protection that had been observed using conventional methods of calculating levels of NK mediated cell lysis (Figure 3.13).

The alternative 'live cell' method of calculation, that was used in the original CIP assay described above, was therefore introduced. This expresses live cell numbers in each condition as a percentage of live cells in the internal control.

$$\left[\frac{\text{live cell numbers}}{\text{live cells in the internal control}}\right] \times 100$$

This method allowed the cells 'lost' from the assay via the debris gate to be accounted for. The same experiment analysed by the two different calculation methods, confirmed that the initial degree of 'protection' observed was equal to the percentage of cells lost from the assay. Despite several repeats, when the 'live cell' calculation method was used, no CIP from NK lysis was observed (Figure 3.14).

The protocol was then modified to include the removal of apoptotic and dead cells via Magnetic Activated Cell Sorting (MACS) following the sub-lytic step. This meant that only live cells entered the second NK killing step (Figure 3.15). When these dead cells were removed, both the conventional and the 'live cell' method of calculating NK target cell lysis produced the same results; this confirmed that sub-lytic complement did not protect cells against lysis by NK cells in these experiments.

3.2.2.4 Timescale of NK Assay

As a 4 hour NK killing assay failed to show any protective effect of sub-lytic MAC, it was possible that this could be due to the length of the assay. CIP has previously been shown to gradually wear off over a period of 8 hours in K562 cells (Reiter, Ciobotariu et al. 1992). This progressive loss of the phenotype means that the

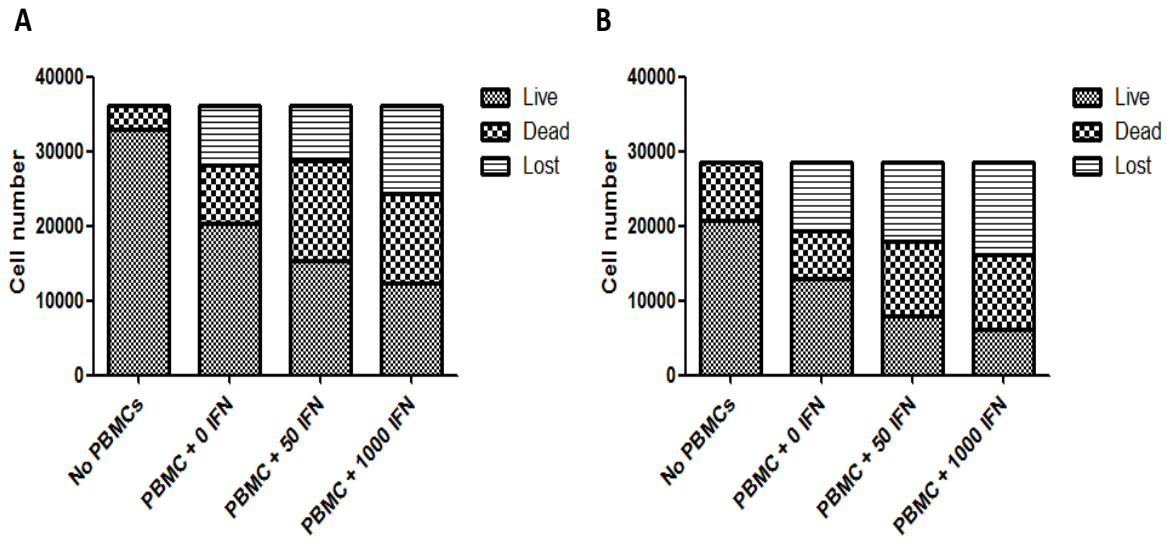


Figure 3.13: Bar charts demonstrating number of live, dead and 'lost' cells. Number of cells 'lost' from the assay was calculated based on the number of cells that were observed in the internal control. Control cells (A), had proportionally more cells visible as dead targets (compared to a no PBMC control), but less cells were 'lost' from the assay. 'Sub-lytically' attacked targets exposed to the same NK assay (B) had more cells 'lost' from the assay. This accounts for differences seen according to calculation methods.

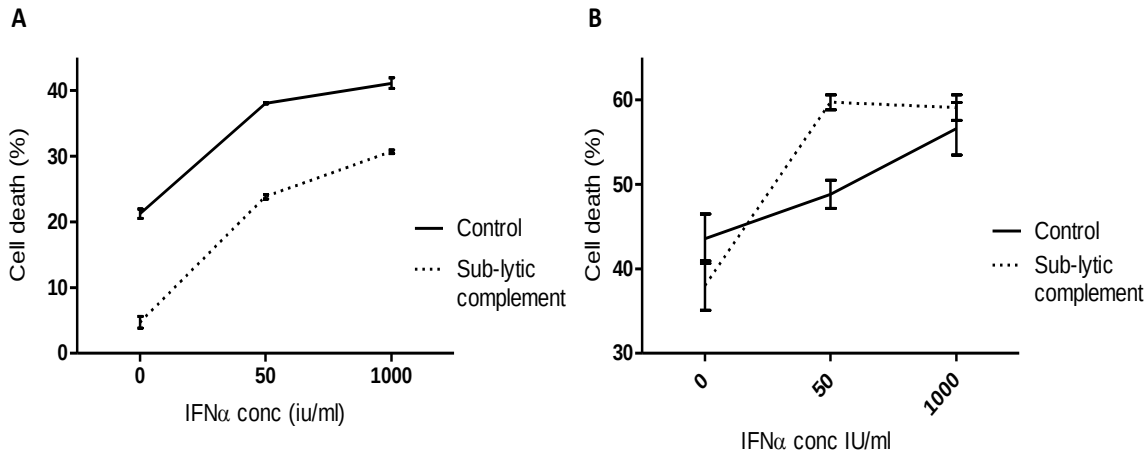


Figure 3.14: Results from same experiment analysed via alternative methods A) experimental results analysed by the conventional method of calculating cell lysis in NK killing assays- (dead targets/total targets) x100 B) the same experiment analysed by (live cell numbers/ live cells in internal control) x 100. This demonstrates that the observed 'protection' is due to a failure to account for complete cell lysis.

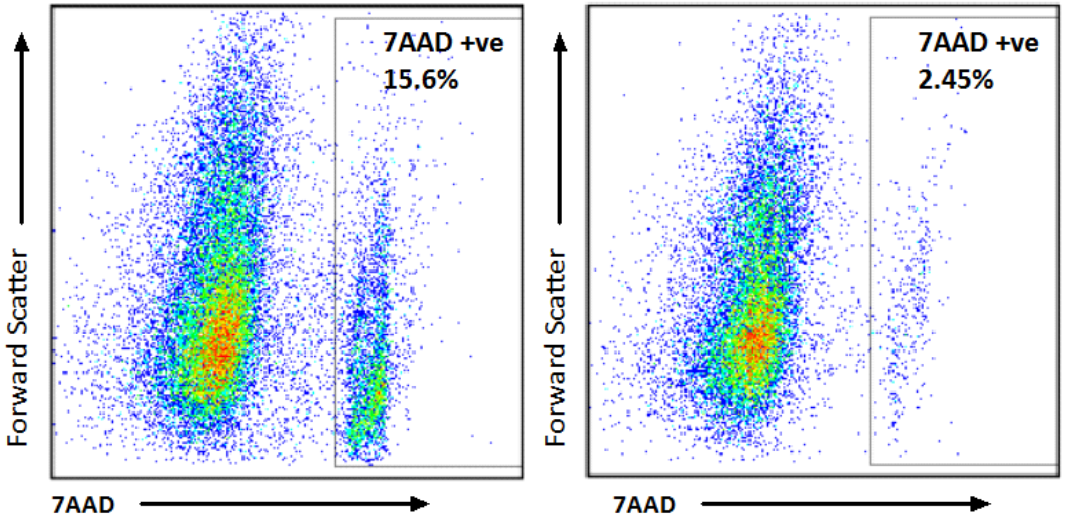


Figure 3.15: MACs removal of dead cells. A dead cell removal kit (Miltenyi Biotec), which recognises an antigen on the surface of both apoptotic and dead cells, was used, in accordance with the manufacturers guidance. It successfully removed dead cells as determined by 7AAD.

protective effect may have been lost during the 4-hour assay. This was explored using time-course experiments to determine whether the duration of the assay could be masking a protective effect. Level of target cell lysis was explored after 1, 2, 3, 4 and 5 hours. No evidence of CIP was observed at any of these time-points (Figure 3.16).

By using a range of PBMC donors, it was shown that NK function was highly donor-dependent with both the extent of NK lysis and the level of target cell lysis over time varying between donors. Some donors had a gradual increase in target cell lysis over time whilst others had similar levels of target cell lysis observed after one hour as seen over the subsequent four hours (Figure 3.17). No protected phenotype was observed using PBMCs from any of the donors.

3.2.2.5 Effector: Target Ratio

Next the effect of altering the E:T ratio was investigated. The original assays utilised an E:T ratio of 5:2, ratios of 2:1, 6:1 and 18:1 were therefore also explored. Despite these modifications, no complement- induced protection from lysis by NK cells was observed (Figure 3.18).

3.2.2.6 Concomitant sub-lytic complement and NK cell attack

The protocol was then modified in order to combine the sub-lytic complement and NK cell killing steps. In this assay, K562 were initially coated with a low dose of TG1 Ab and then exposed to NK cells in media in which the 10% FBS was replaced with 10% NHS (Figure 3.19). This resulted in a sub-lytic complement attack occurring concomitantly with the NK killing assay. The assay was explored at a range of E:T ratios. These assays again failed to demonstrate any evidence of CIP from NK killing (Figure 3.20).

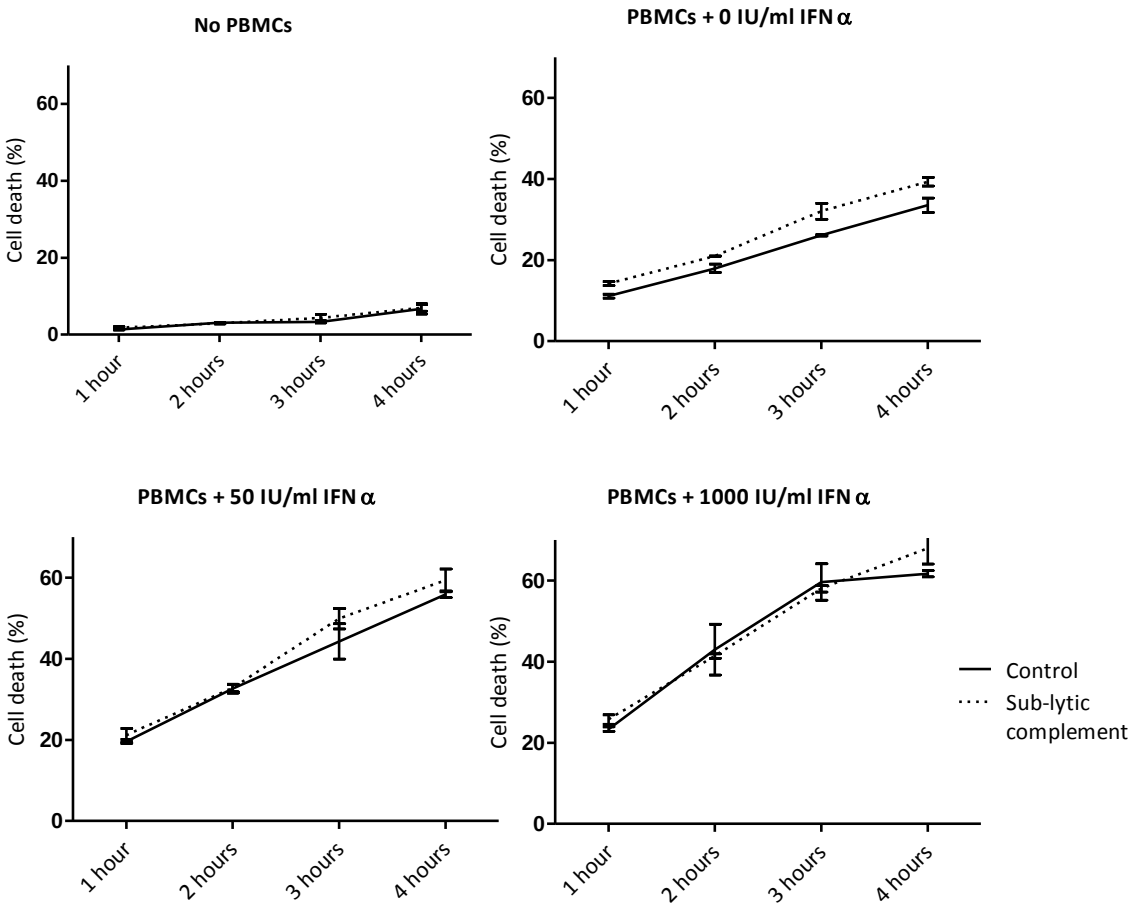


Figure 3.16: Cell death after 1, 2, 3 and 4hrs. Samples were analysed at hourly timepoints to assess for level of cell lysis. PBMCs preconditioned overnight with 0, 50 or 1000IU IFNα were used as effectors at an E:T ratio of 5:2. No evidence of sub-lytic complement offering a protective effect was observed at any time point in any of the conditions tested.

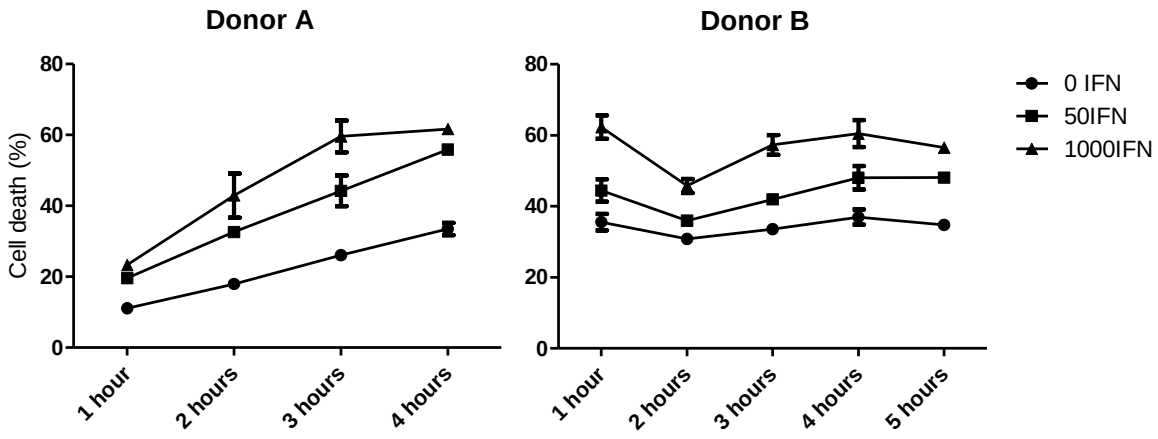


Figure 3.17: Target cell lysis over a 4/5 hr time-course in 2 normal donors. PBMCs preconditioned overnight with 0, 50 or 1000IU IFNα were used as effectors at a E:T ratio of 5:2.PBMCs from donor A resulted in a gradual increase in target lysis over the 4 hr time-course peaking at ≈ 60% cell death. Donor B had a similar level of target lysis at one hour as observed over the subsequent 4 hours. These findings were confirmed on repeat analysis.

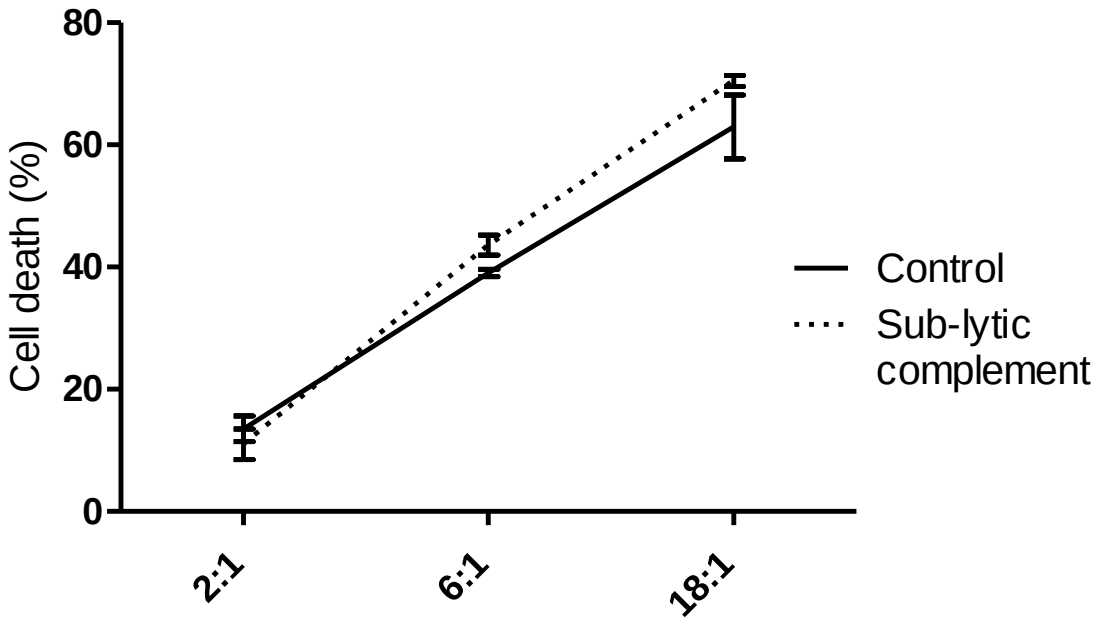


Figure 3.18: Cell death as a range of effector: target ratios. K562 exposed to sub-lytic complement had similar level of target lysis as control targets when exposed to PBMCs conditioned overnight with 50IU IFNα at E:T ratios of 2:1, 6:1 and 18:1.

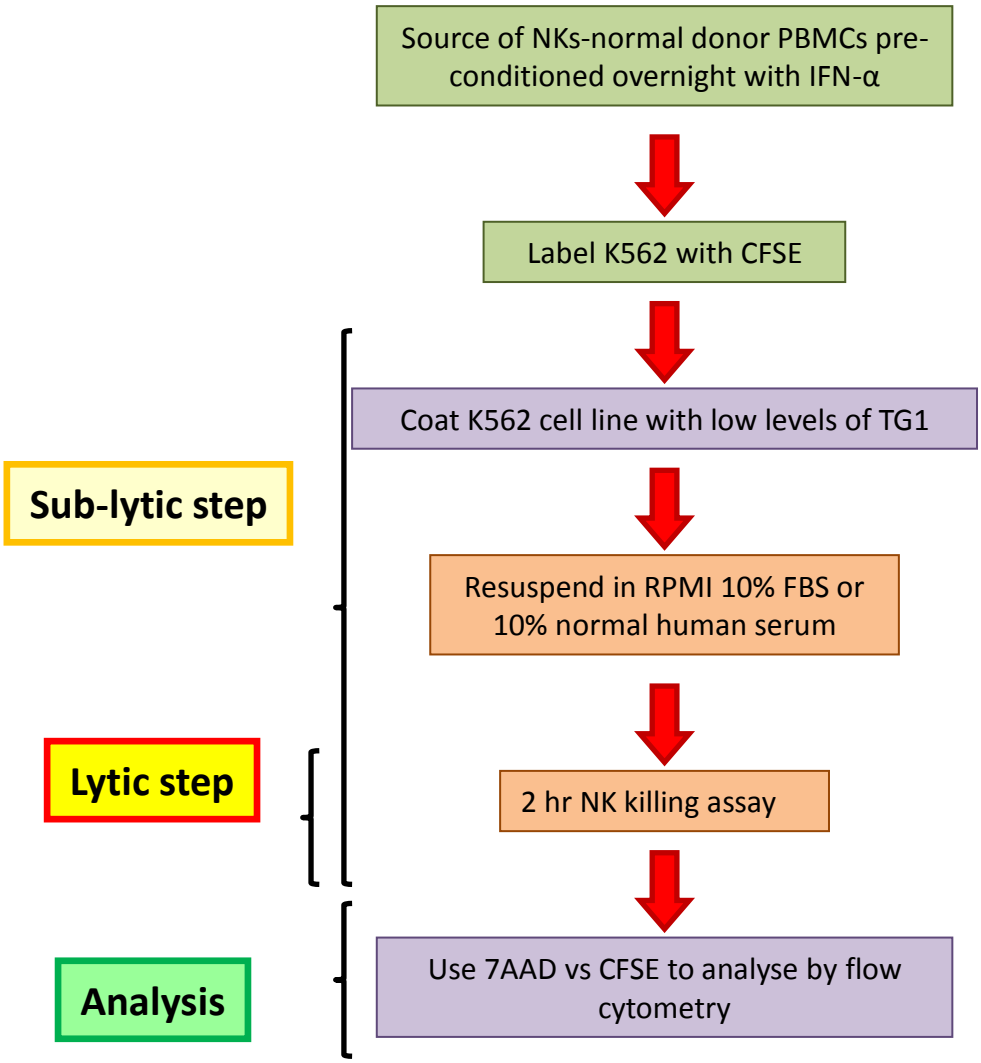


Figure 3.19: Schematic showing an assay combining sublytic complement attack with the NK killing assay. In this assay the 10% foetal bovine serum present in the media used during the NK assay was substituted with NHS which allowed TG1 coated target cells to be attacked by a sublytic dose of complement and NK cells simultaneously.

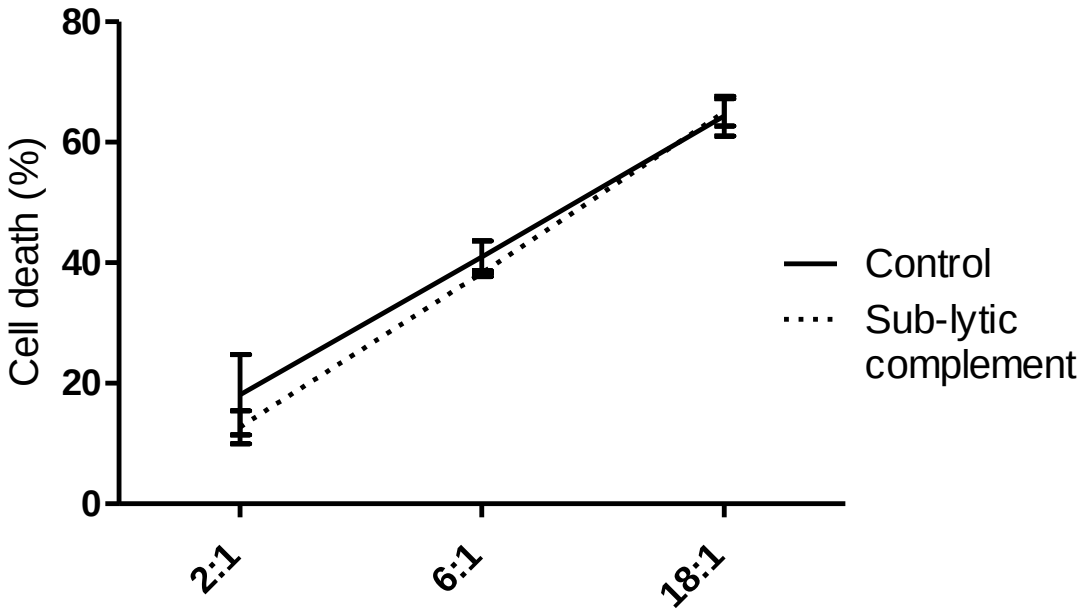


Figure 3.20: Combined sub-lytic complement and NK killing assay. TG1 coated and control K562 were exposed to PBMCs conditioned overnight with 50IU IFNα at E:T ratios of 2:1, 6:1 and 18:1 in an assay which contained 10% NHS as a source of complement. Thus a sub-lytic complement and NK attack were occurring simultaneously. This assay revealed no evidence of CIP from NK killing at a range of E:T ratios.

3.2.3 Determine a Genetic Signature for Complement-Induced Protection

As demonstrated earlier in this chapter, exposure to a sub-lytic dose of complement induces a high level of protection from subsequent lytic attack. Although it is known that the CIP is dependent on the assembly of the complete MAC, the presence of extracellular Ca^{2+} and on active cellular metabolism, the exact mechanism underpinning it remains unknown. In order to explore potential mechanisms, a transcriptomic analysis was performed to determine whether protection is accompanied by genetic changes within protected cells.

3.2.3.1 Experimental Design

Experimental design is critical in order for data analysis to arrive at a statistically and biologically valid conclusion. Experimental plans were therefore discussed with a bioinformatician and a biostatistician prior to embarking on the experiment. It was agreed that biological replicates would offer better data than technical replicates alone. Design is a balance of resources, accuracy and reliability. It was generally felt that large differences in gene expression could be detected with 3 samples per condition whereas smaller differences might require 5 samples per condition. Due to the range of controls required, there were 5 experimental conditions included in this study, meaning that cluster analysis would be possible. In order to perform clustering analysis it is generally perceived that more than 20 samples are required. It was therefore decided that two Illumina beadchips would offer the ability to analyse 24 RNA samples, derived from 4/5 experimental repeats, simultaneously.

Figure 3.21 gives an overview of the experimental protocol and illustrates the checks that were in place to confirm the validity of each experiment repeat. A great deal of care was taken to standardise experimental conditions such as RNA stabilisation and extraction techniques. K562 were passaged the day prior to each experiment such that they were in fresh media and were not over-confluent. Fresh cells were thawed from the same batch of frozen K562 stock at regular intervals such that the passage number was the same. As complement attack required

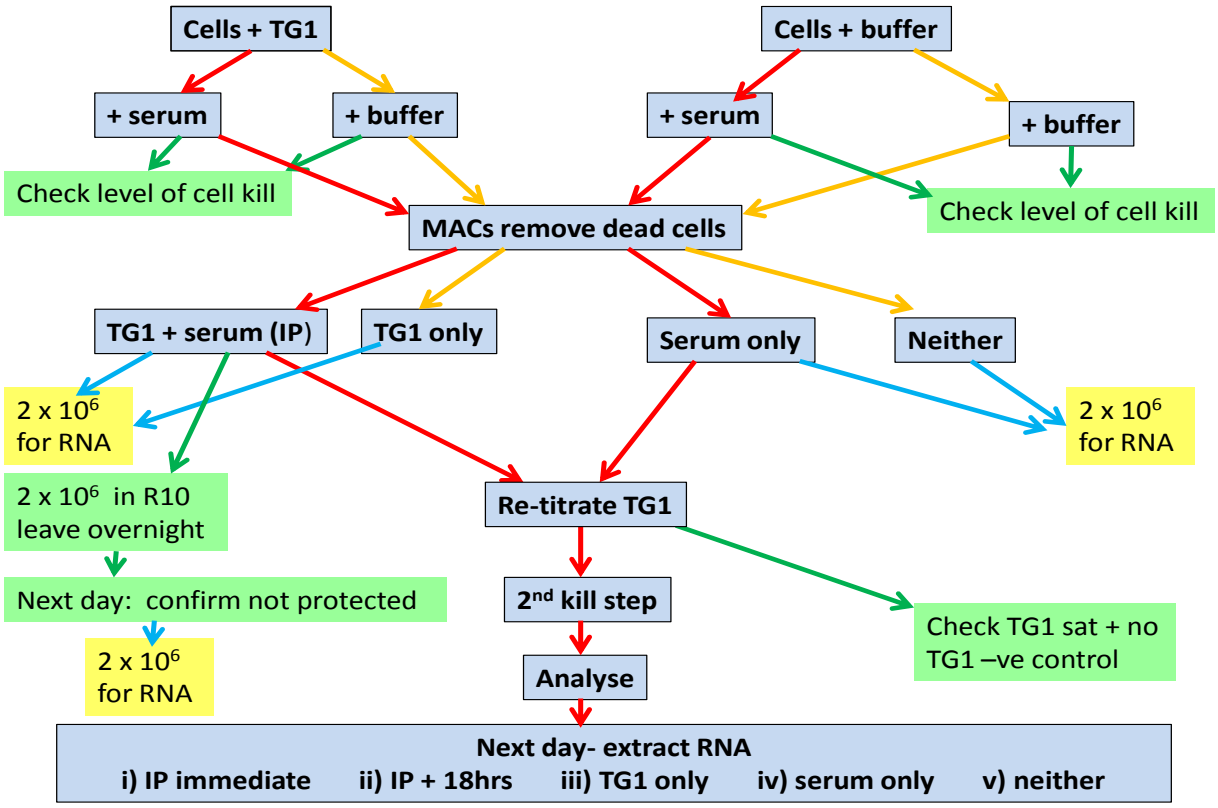


Figure 3.21: Protocol for acquiring and assessing samples for RNA extraction. K562 cells were exposed to titrated doses of TG1 and NHS, resulting in 5-10% complement mediated lysis. Dead cells were then removed using Magnetic Activated Cell Sorting. RNA was extracted from 2 x 10^6 protected cells (IP) and control cells which had been exposed to either Ab alone, NHS alone or neither. The remaining cells then underwent a 2^{nd} kill step to confirm that cells had a strong 'protected' phenotype. Protected cells were also allowed to recover overnight before extracting RNA from a further 2 x 10^6 cells as an additional control group.

exposure to both Ab and NHS, controls which had been exposed to an equivalent dose of either NHS (SC) or the TG1 Ab (TC) were included in the experiment along with cells which had been exposed to neither (C). An additional control was included which contained RNA from cells which had been exposed to sub-lytic MAC but had been allowed to recover overnight (ON) and were therefore no longer 'protected'. Several additional checks were put in place to confirm that each experiment was viable.

Following the sub-lytic attack, a sample of cells taken from each condition was stained with 7AAD and fixed in order to confirm that the first complement attack had resulted in the correct level of cell kill (5- 10%) (Figure 3.22). In order to prevent interference from the presence of apoptotic cells, a MACS kit which recognises and removes both apoptotic and dead cells was also used prior to RNA extraction. 2 x 10^6 cells from each test condition were then immediately submerged in RNAlater Stabilization Reagent and stored at 4°C. RNAlater rapidly permeates cells, stabilising the RNA and preserving the RNA expression profile. This prevents changes to the gene expression profile occurring due to RNA degradation and gene induction and down-regulation, which can be triggered by sample manipulation. This ensures that downstream analysis accurately reflects the expression profile of the cells.

TG1 Ab was then re-titrated on both the remaining IP and SC cells. A sample of these cells were stained for surface TG1 binding and fixed so that levels could be confirmed to be equivalent/ slightly lower on the SC cells such that any bias was in favour of control cells (Figure 3.23). TG1 coated cells were then exposed to a range of NHS dilutions so that a survival curve for both the IP and SC cells could be plotted to confirm protection (Figure 3.24).

Some protected cells were allowed to recover in the incubator overnight. The following day, 2 x 10^6 of these cells were also stored in RNAlater. TG1 was then titrated on the cell surface and a further exposure to lytic doses of complement confirmed that cells were no longer protected (Figure 3.25).

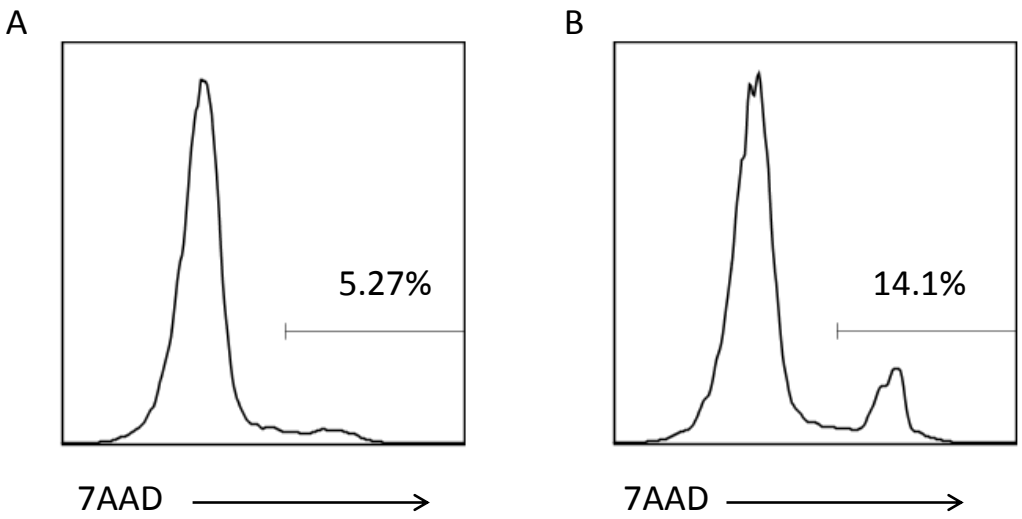


Figure 3.22: Cell death post sub-lytic complement. A) control cells demonstrated 5.27% 7AAD +ve cells whereas cells exposed to a sub-lytic dose of complement (B) were 14.1% 7AAD +ve resulting in a cell death above baseline of 8.83% (target 5-10%).

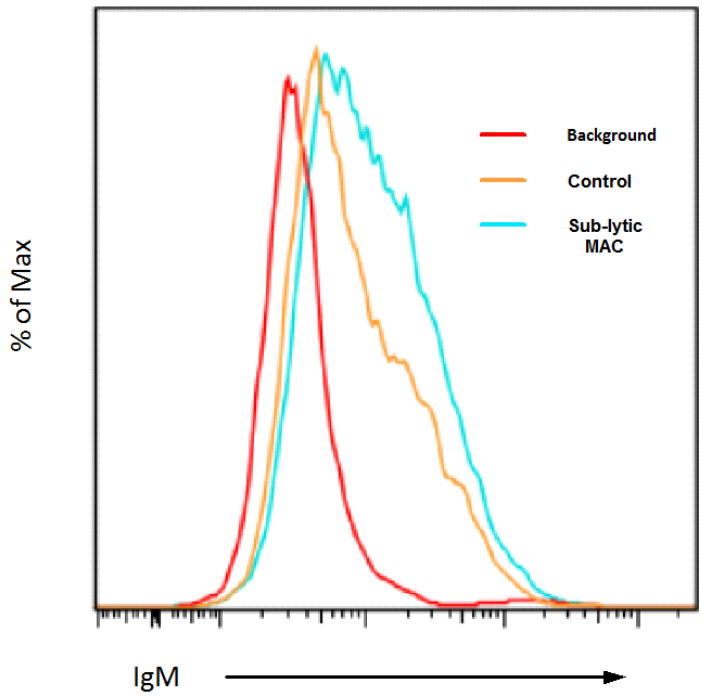


Figure 3.23: Levels of surface IgM (TG1). Following 2nd incubation with the anti-myeloid TG1 Ab, levels of Ab were assessed by measuring levels of surface IgM using a biotynylated anti-IgM Ab conjugated to strepavidin PE. In each case, a control with a slightly lower level of surface TG1 was chosen as a comparator, such that any bias was in the favour of the control.

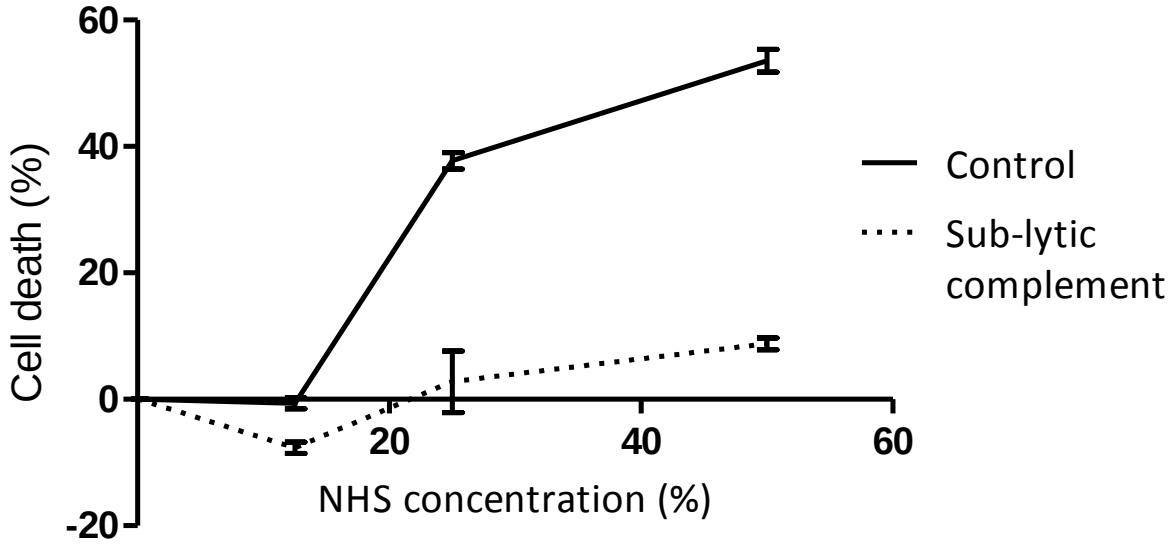


Figure 3.24: Killing curves confirming the presence of complement-induced protection. Cells exposed to a sub-lytic dose of complement were protected from a lytic dose of complement when compared to control cells that had a prior exposure to complement.

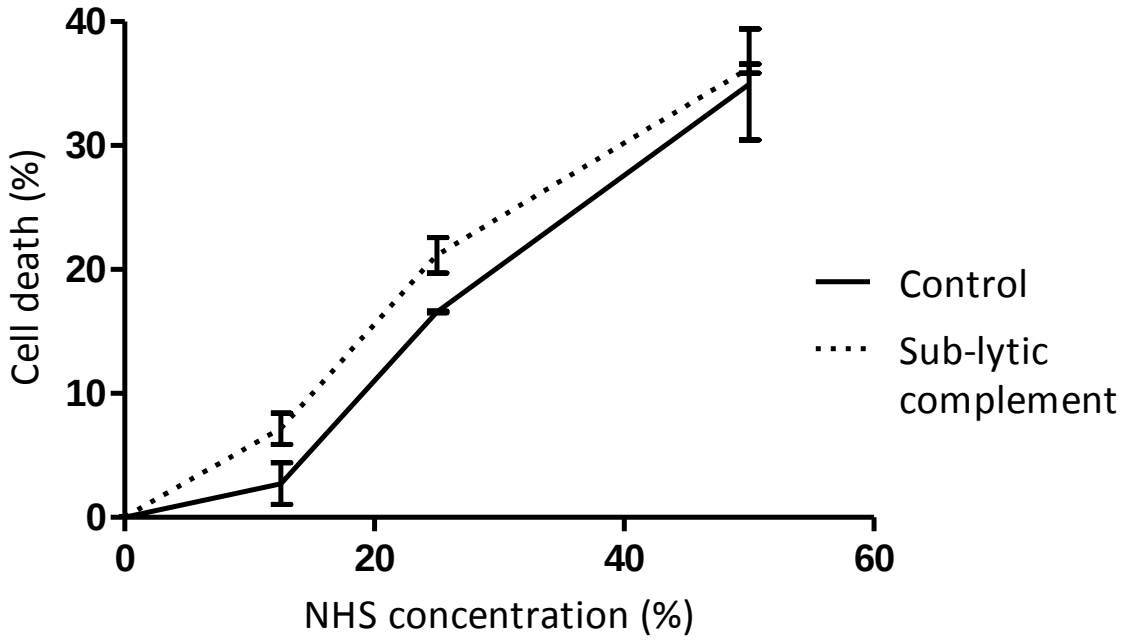


Figure 3.25: Killing curves confirming that complement-induced protection is a time-limited phenotype. Sub-lytically attacked cells were allowed to recover overnight before being re-incubated with TG1 Ab and exposed to a lytic dose NHS confirming that cells had lost their protected phenotype.

When each of the following three checks were met: i) correct level of sub-lytic attack ii) equivalent surface TG1 iii) protection from lytic complement, RNA was then extracted. A Qiagen RNeasy Mini Kit was used to extract RNA from both the cells that had recovered overnight (ON) and the cells from the other conditions (IP, SC, TC, C) which had been stored in RNAlater overnight. An aliquot of the resulting eluted RNA was used to measure the amount of RNA present using a spectrophotometer (nanodrop).

Samples from four complete experiments were included in the microarray. In order to maximise use of the chips 4 additional RNA samples were also included resulting in inclusion of 24 RNA samples extracted from: 7 IP, 5 TC, 4 SC, 4 C and 4 incubated overnight (ON) cell samples (summarised in Table 3.1).

	IP	**SC**	**TC**	**C**	**ON**
Experiment 1	X 2	X 1	X 1	X 1	N.I
Experiment 2	X 2	X 1	X 2	X 1	X 2
Experiment 4	X 1	N.I	N.I	N.I	N.I
Experiment 5	X 1	X 1	X 1	X 1	X 1
Experiment 6	X 1	X 1	X 1	X 1	X 1

Table 3.1: RNA samples taken forward for microarray analysis. IP = induced protection, SC = NHS control, TC = TG1 control, C = untreated control and ON = incubated overnight. N.I = not included.

All extracted RNA included in the analysis was shown to be of high quality with only minimal degradation (Agilent 2100 Bioanalyzer). An Illumina Human HT-12v4 BeadChip was used to probe for mRNA expression. BeadChips use oligonucleotides immobilized to microscopic beads held in microwells on the surface of array substrate. Each array targets 47,231 probes, covering well-characterised genes, gene candidates and splice variants and is able to analyse twelve samples leading to reduced sample to sample variability.

3.2.3.2 Microarray Analysis

As microarray data sets are extremely large they offer significant statistical challenges. It is essential to take into account effects of background noise and to use appropriate normalization of the data. Analysis was completed in collaboration with a Bioinformatician, Robert Andrews. The code an007.R was used to run the analysis. The lumi and limma packages in BioConductor were used to analyse the data. Raw data was inputed from GenomeStudio with replicate probes on the array being averaged to a single value per probe. Normalisation of data within an array controls for systematic biases introduced by factors such as variable dye coupling or hybridisation efficiencies allowing true biological differences to be found. For the purpose of comparing datasets, data were normalised using Variance Stabilizing Transformation (VST) (the standard method for Illumina gene expression arrays) where data are background subtracted, VST transformed and quantile normalised.

3.2.3.3 Quality control

Box plots of both raw data and quantile-normalised data were used to assess the quality of the data. These illustrate the distribution of spot intensities on the array with any sample having a disproportionately slim or wide box, in the context of all other samples, indicating an issue. My arrays resulted in uniform box sizes illustrating good consistency between spot intensities on the arrays, confirming the quality of my data to be good (Figure 3.26).

3.2.3.4 Class Discovery Analysis

Class discovery analysis or unsupervised classification aims to identify whether microarrays cluster together in groups and whether these have biological significance. This classification is not hypothesis-driven, but uses pattern recognition to find an optimal number of clusters in the data. In this study hierarchical cluster analysis and principal component analysis were used to assess

the natural clustering of the experimental groups. Biological replicates would be expected to cluster together within the plot.

An initial Hierarchical Clustering analysis revealed that samples were not grouping by experimental group but appeared to be more closely aligned to the experimental batch they had arisen from (Figure 3.27). A principal component analysis also confirmed this observation (Figure 3.28).

3.2.3.5 Hypothesis-Driven Statistical Analysis

Hypothesis-driven statistical analysis was then carried out in order to identify statistically significant changes in gene expression unique to the IP condition. Analysis of data demonstrated no differentially expressed genes unique to IP. This was initially analysed using pair-wise comparisons which, although giving a short list of differentially expressed genes between IP and TC and C, failed to show any differences between the IP and SC. As both the IP and SC conditions had been exposed to NHS, this suggests that the expression changes seen between the other pairings (where in each case one had and one had not been exposed to NHS) was likely to be due to a serum effect. In line with this, on review of the hierarchical clustering dendrogram, it was noted that IP and SC conditions were generally paired together.

Bioreplicates not clustering together could indicate several things:

(i) accidental sample swaps/mislabelling
(ii) differential gene expression is only slight relative to the differences observed between the biological replicates- i.e it is a noisy system
(iii) the list of significant genes is small (1-50 genes)

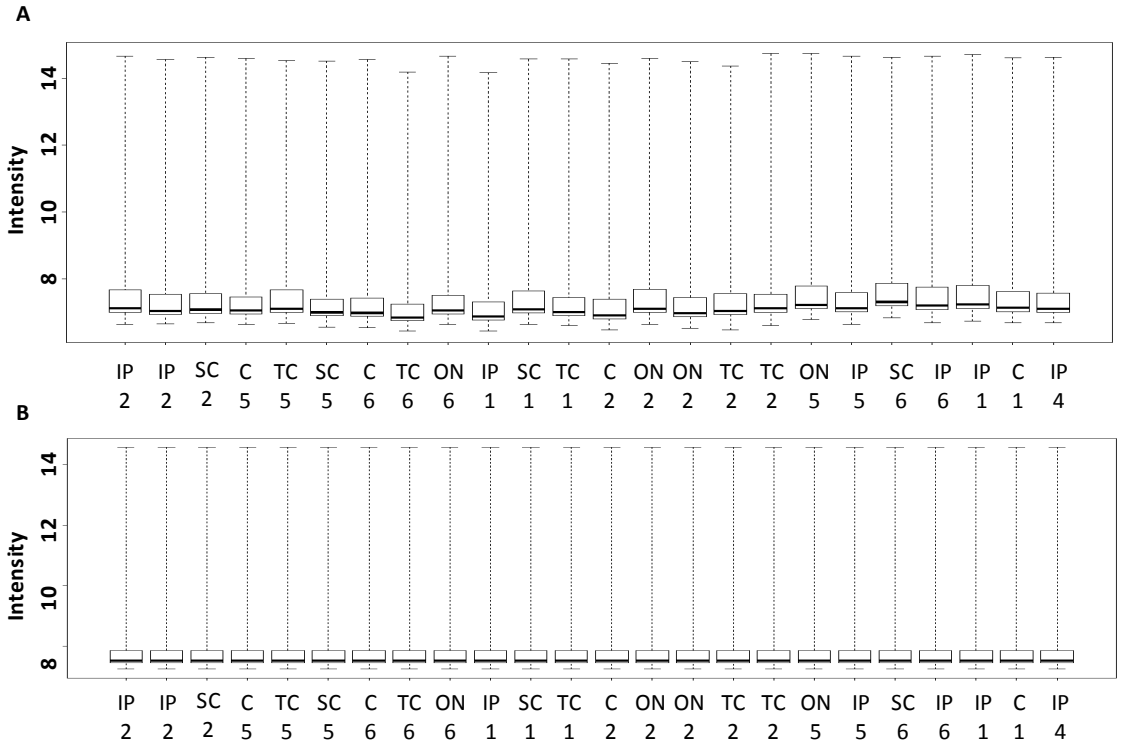


Figure 3.26: Box plots showing microarray intensity of both A. raw data and B. quantile-normalised data: box plots are used to assess the quality of the data by demonstrating the distribution of spot intensities on the array. No sample was identified to have a disproportionately narrow or wide box. It was concluded that the quality of the RNA samples analysed was good.

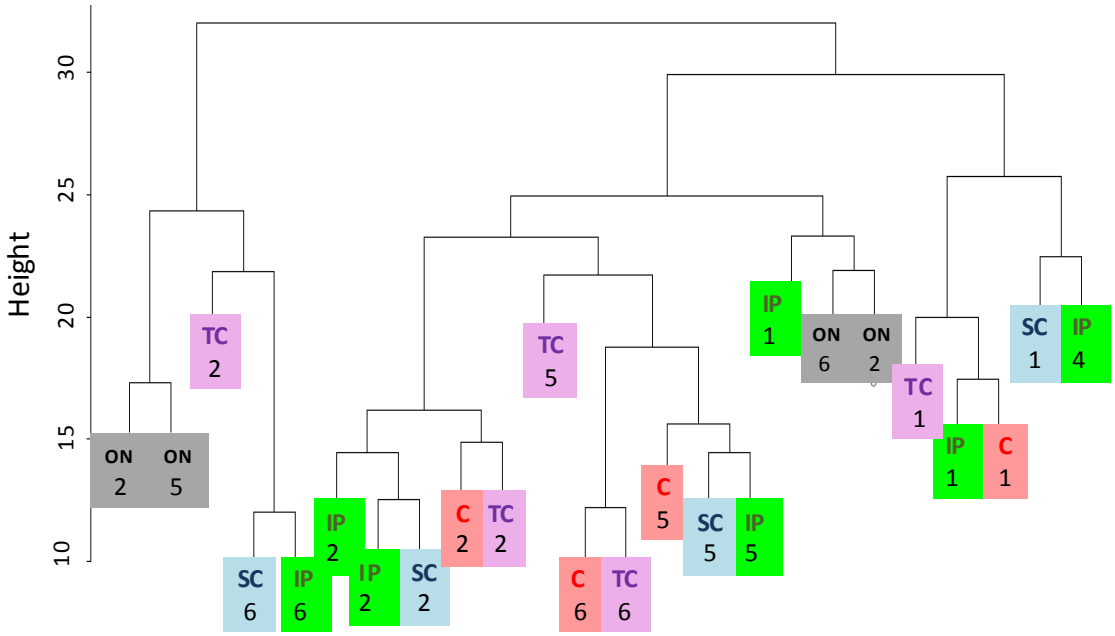


Figure 3.27: Hierarchical Clustering of samples. The dendrogram shows the relatedness of data based on 24,848 genes with SD/mean >0.1. Green/IP = induced protection, blue/SC = NHS control, purple/TC = TG1 control, red/C = untreated control, grey/ ON = recovered overnight. Number specifies which experimental repeat the RNA sample originated from.

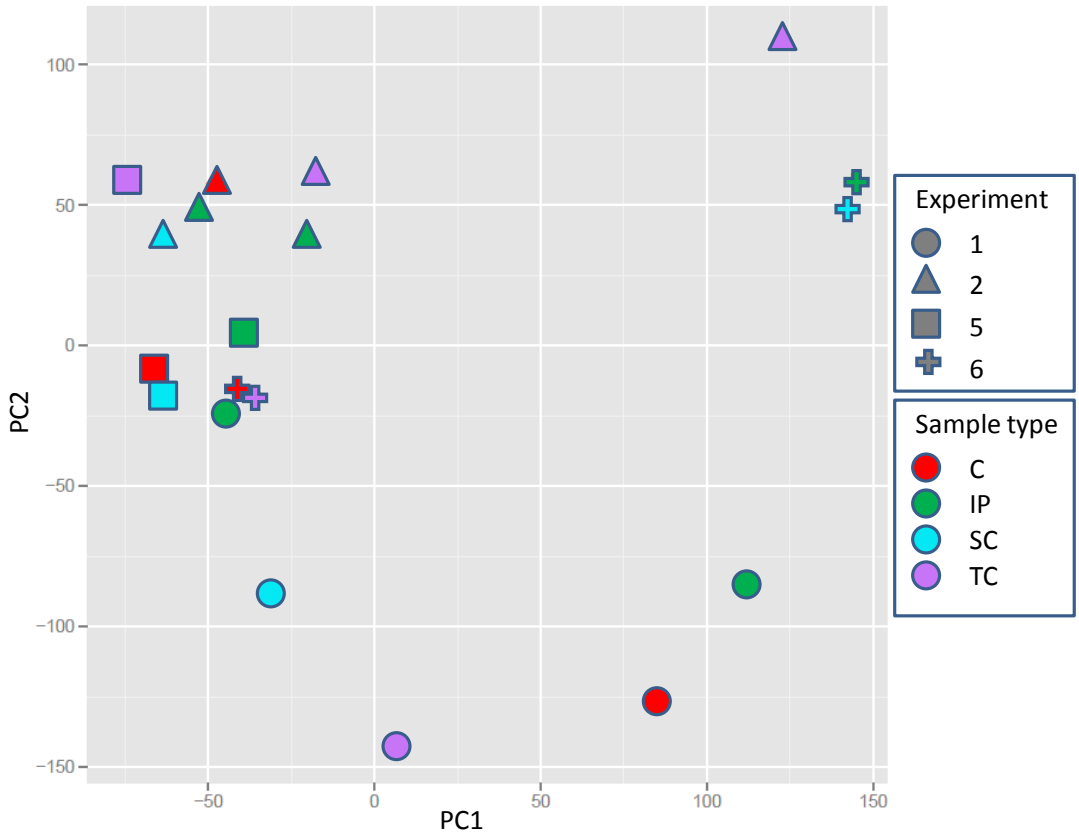


Figure 3.28: Principal component analysis. The relatedness of gene expression data is demonstrated using a principal component analysis based on 24,848 genes with SD/mean >0.1. Green/IP = induced protection, blue/SC = NHS control, purple /TC = TG1 control, red/C = untreated control. Only experiments 1, 2, 5 and 6, which had a complete set of controls, were included in this analysis. This alternative method of unsupervised classification again demonstrated data to group far more by experimental repeat (defined by shape) than by biological replicate (defined by colour).

The multiple checks in place, consistency of experimental grouping and different days on which experiments took place made accidental swapping/ mislabelling an unlikely explanation. The consistency of cell passage, experimental repeats and controls meant that the system was as consistent as possible.

3.2.3.6 Result Validation

The following methods were used to validate these findings:

(i) changing the normalisation method
(ii) building a batch effect into the model
(iii) removing potential outliers from the analysis

3.2.3.6.1 Changing the Normalisation Method

Where an analysis involves comparisons between 3+ samples (in this case ≥4 samples), normalisation of data typically follows a method of normalising all data together. This method is preferred for the reason that an outlier array (one that behaves slightly differently) will be normalised in line with the bulk of "good" data. This method fails where an outlier array behaves very differently from all other data, and the array therefore 'poisons' the good data of other arrays.

In this study both "all array" normalisation and pair-wise normalisation were performed and compared. Pair-wise normalisation in this case means that for the comparison of A vs B only data for arrays A and B were normalised with respect to each other. No differentially expressed genes were observed whether data were normalised by an "all array" or a "pair-wise" method.

3.2.3.6.2 Building a Batch Effect into the Model

The batch effect seen within the hierarchical cluster would likely be observed if (i) there was a significant batch effect; (ii) there was no biological effect to be

observed and the act of "zooming into" the data showed a batch effect. The analysis was performed both with and without a batch correction, for both normalisation methods. Again, no differentially expressed genes were observed. This suggests there is no biological effect and that the observed 'batch effect' was the result of over amplifying small non-significant differences between experiments.

3.2.3.6.3 Identify Potential Outliers and Remove from the Analysis

Potential outliers were identified by examining the hierarchical clustering analysis. These were removed individually and in combination and the residual samples were analysed for differentially expressed genes. No differentially expressed genes were observed.

3.3 Discussion

Results described in this chapter show that CIP is a convincing, reproducible phenomenon. Whilst this has previously been demonstrated using chromium release assays, I have shown that CIP can also be demonstrated using a flow cytometric based assay.

Previous studies using C7 and C8 deficient serum have proved that it is the presence of MAC itself, as opposed to any earlier components of the complement cascade, which leads to the protective phenotype (Reiter, Ciobotariu et al. 1992), however the exact mechanism remains unclear. The 'protected' phenotype is immediate and very reproducible. Although aiming for a target of 5-10 % cell death, I noted that even when this level was exceeded, surviving cells were always and often even more convincingly protected. This suggests that high surface levels of MAC, as long as they don't result in immediate cell lysis, lead to a profound protective effect from subsequent complement attacks.

The level of protection was also noted to be most convincing when tested for immediately, with delays resulting in a lower level of protection being observed.

The inclusion of additional steps, such as MACS removal of dead cells, whilst improving the data in some respects and allowing use of conventional methods of calculating cell death, led to a less profound protective phenotype, most likely due to the delays ensued. This is supported by the literature with 'protected' cells being shown to become gradually sensitive to lytic complement doses over time (Reiter, Ciobotariu et al. 1992). K562 cells start removing MAC from their surface within 5-10 minutes of complement attack by outward and inward vesiculation (Moskovich and Fishelson 2007).

Despite investigating multiple variations to my experimental protocol including timescale analysis, altering E:T ratio, using alternative PBMC donors and developing an alternative combination assay, no convincing evidence of *in vitro* CIP from NK cell attack was observed during my studies.

This could be because K562 cells are extremely susceptible to NK mediated lysis and sub-lytic complement attack is not sufficiently protective to offer any effect. Future experiments should revisit this system with as minimal intensity as possible via removing any IFNα pre-conditioning and using an even lower E:T ratio. However, my studies thus far have offered no evidence to suggest that this approach would prove successful. As K562 was used in the original studies describing CIP and is widely use in the study of NK biology, it was the obvious choice of cell line to be used in these studies. However, a cell line which is less susceptible to NK cell lysis might offer more insight into the role of CIP in this setting. Alternatively, K562 could be altered in order to make it less susceptible to NK lysis via methods such as increasing its expression of MHC class 1 (Kaufman, Schoon et al. 1995).

As previous studies used perforin purified to homogeneity from the granules of NK cells (Reiter, Ciobotariu et al. 1995), it could also be the case that sub-lytic complement attack does not offer protection from whole cell NK attack, which utilises a range of mechanisms .

Whilst complement relies on MAC to disrupt cell homeostasis and lyse cells, NK cells mediate cytotoxicity via a range of distinct mechanism. The most studied mechanism is degranulation which utilises perforin in order to form the pores through which inducers of cell death such as granzymes are introduced into target cells. Degranulation occurs following the formation of an immunological synapse which is the result of complex interaction between activating cell surface receptors counteracted by signals from inhibitory receptors, most of which bind to MHC class 1 molecules (Bryceson and Long 2008). A sub-lytic complement attack is likely to result in cellular stress. Cellular stress, from physical, pathogenic or oncogenic sources, leads cells to down-regulate MHC class 1 and up-regulate ligands for NK activating receptors, resulting in them becoming more susceptible to NK attack (reviewed in (Chan, Smyth et al. 2014)). This stress response could be compensating for any protection provided by CIP against the insertion of perforin into the cell membrane. Thus more activated NK cells forming immunological synapses could be compensating for their hampered ability to form cytolytic pores. This would only be relevant in a whole cell assay and might explain why CIP was not observed in this model as opposed to observations from earlier studies which utilised purified perforin.

NK cells also exert their cytotoxic effects on target cells through death receptor pathways such as the TNF-related-apopotosis-inducing-ligand (TRAIL)–TRAIL and Fas-FasL pathway. Death receptors induce apoptosis via caspase activation thus inducing cytotoxicity independently of NK cell degranulation. The impact of complement on TRAIL and FAS pathways has not been explored but might be an alternative mechanism through which cells exposed to complement are being killed.

As CIP has been shown to be dependent on both protein and RNA synthesis (Reiter, Ciobotariu et al. 1992), it was reasonable to assume that it would result in a unique gene expression phenotype or ‘signature’. Perhaps somewhat surprisingly, comprehensive microarray analysis revealed no significant gene expression changes. Finding no differentially regulated genes is an unusual event during microarray analysis, where the more usual concern is having too long a list of

dysregulated genes. A range of confirmatory steps were therefore performed along with a review of the data by a second Bioinformatician, all of which supported our original conclusions.

A strong 'protected' phenotype was confirmed to be present in all sub-lytically attacked cells from which RNA was extracted. This, along with the fact that we could clearly observe a 'serum effect' in RNA extracted from samples exposed to NHS, strengthens the case that if the protected phenotype was due to alterations in gene transcription, this would have been detected. This raises the possibility that CIP is due to an alternative underlying mechanism.

As discussed above, during my studies I observed that the 'protected' phenotype was strongest immediately following a sub-lytic attack. This is supported by previous data demonstrating that cells gradually became susceptible to lytic complement over the 8 hours following a sub-lytic attack (Reiter, Ciobotariu et al. 1992). Within minutes of complement attack polymorphonuclear cells repair their membranes, approximately 35% via endocytosis and 65% via membrane shedding (Morgan, Dankert et al. 1987). The insertion or shedding of membrane attack complexes could result in structural or compositional change to the cell membrane, or underlying scaffolding elements, which could prevent MAC or other pore-formers from being inserted into the cell membrane.

The preferential loss of cholesterol-rich regions of the cell plasma membrane, termed lipid rafts, could impinge on subsequent attack. The lipid raft hypothesis suggests that the lipid bilayer is not structurally passive but instead organises itself into sub-compartments of cell membrane which have specific bioactivity (Lingwood and Simons 2010). Lipid rafts are dynamic, nanoscale assemblies enriched in sphingolipid, cholesterol and GPI anchored proteins which form functional platforms (Hancock 2006). Ab cross-linking at the cell surface leads to raft lipids and proteins co-localising and excluding non-raft proteins (Harder, Scheiffele et al. 1998). The assembly of lipid rafts is always cholesterol dependent and some studies have demonstrated a requirement for the rearrangement of actin (Suzuki, Fujiwara

et al. 2007; Goswami, Gowrishankar et al. 2008). The enrichment of GPI anchored proteins, such as the complement regulators CD55 and CD59, in lipid rafts (Brown and London 2000) suggests that they could be a target for complement attack. The ability to induce lipid rafts also leads to the activation of complement dependent cytotoxicity (Cragg, Morgan et al. 2003). If MAC is preferentially inserted into these lipid rich areas, a sub-lytic complement attack would not only lead to shedding of membrane vesicles containing MAC, but could also result in the loss of lipid rafts. Loss of these cholesterol rich areas could impinge on the ability of MAC or other pore-formers to insert into the cell membrane until lipid rich areas are replenished.

Alternatively, re-configuration of the actin skeleton, a mechanism utilised by pathogens such as cytomegalovirus (CMV), could be relevant. Certain strains of CMV have evolved immune evasion genes which protect the host cell from NK and T cell attack. This protection is provided via remodelling the actin skeleton resulting in a dramatic reduction in the efficiency of immune synapse formation (Stanton, Prod'homme et al. 2014). Whilst cytotoxic cells are known to reorganise their actin skeletons in order to promote adhesion, polarization of cytotoxic granules, organisation of the immunological synapse and degranulation, there is surprisingly little data regarding the role of the actin cytoskeleton in target cells. The ability of actin reorganisation in target cells to influence synapse formation could also affect the ability of pore-formers to insert into the cell membrane making this a potential mechanism through which sub-lytic complement attack might result in resistance from further pore-forming attack.

Concluding remarks

Whilst sub-lytic complement offers convincing protection against a subsequent lytic complement attack, I have failed to demonstrate any convincing protection against NK cell killing. This does not however, rule out the possibility that CIP is a mechanism at play during NK cell attack. This could be due to failings in methodology such as the use of a cell line that is highly susceptible to NK mediated cell lysis or alternative mechanisms, such as the cellular stress caused by a

complement attack, could be compensating for the protective effect. Further studies are required in order to investigate these mechanisms.

Despite a strongly protective phenotype, I have also failed to demonstrate a genetic signature for CIP. Comprehensive analysis and confirmatory steps have shown no dysregulated genes unique to CIP despite the ability to identify a genetic signature for other experimental variables such as exposure to NHS. This strongly suggests that an alternative mechanism is involved. I have discussed two potential mechanisms that could be significant and believe that further studies involving actin imaging and lipidomic investigation of cell membranes following complement attack are warranted. As previous studies suggest that this phenomenon is not unique to the complement system, it would also be informative to explore a range of pore-formers including MAC, perforin, melittin and streptolysin.

Chapter 4- Complement promotes progression of AML through multiple mechanisms

4.1 Introduction

The complement system is activated in a range of malignancies (discussed in Chapter 1, reviewed in Table 1.4) with the level of activation often being proportionate to disease progression (Nishioka, Kawamura et al. 1976; Matsutani, Suzuki et al. 1984; Guidi, Baroni et al. 1988; Niculescu, Rus et al. 1992). Production of the complement anaphylatoxin C5a can create a favourable microenvironment for disease progression by impinging on CD8+ T cell responses (Markiewski, DeAngelis et al. 2008). C5aR expression has also been shown to be a feature of metastatic spread (Vadrevu, Chintala et al. 2014).

AML cells exert a range of effects on the immune system in a bid to evade immune elimination (reviewed in Chapter 1). However, the effect of the complement system on the progression of AML has not as yet been explored. The complement system is activated in AML, with levels of complement proteins falling once a remission is attained (Minh, Czink et al. 1983). More recent studies have revealed a potential role for the complement protein C3f-desArg in both diagnosis and detection of minimal residual disease (MRD) (Liang, Wang et al. 2010) in acute leukaemia, suggesting that the complement system could be activated during the earliest stages of disease development and relapse. This led me to postulate that AML cells activate the complement system in order to evade immune elimination.

Immune therapies are already widely used in the treatment of AML, with novel agents constantly being investigated and developed for use. If complement has a role in the progression of AML, then complement-targeted therapeutics may not only have an independent role in improving spontaneous immune responses to leukaemia, but may also impact on all therapies aimed at controlling AML via immune mediated mechanisms.

The main aims of experiments described in this Chapter were to:

- Investigate whether complement activity affects the progression of AML
- Determine which components of the complement cascade are key
- Explore the underlying mechanisms.

Studies outlined in this chapter used the mouse AML cell line, C1498, which was originally cultured *in vitro* from AML which developed spontaneously in a B6 mouse. The C1498 cell line used expressed the fluorescent protein DSRed which enables detection of the tumour within the tissue *in vivo* (Sauer, Ericson et al. 2004). In my studies, progression of AML was monitored *in vivo* using Kodak FX Pro fluorescence imaging equipment (FX Pro).

4.2 Results

4.2.1. Disease developing post IV administration of C1498FFDsR is detectable using Kodak FX Pro fluorescence (FX Pro) imaging equipment

Initial studies assessed the dose of C1498FFDsR which would result in consistent take and progression of AML. Based on available literature, a dose of 10^6 cells was initially compared with a higher dose of 5 x 10^6 cells. Cells were administered IV to three immunocompetent female WT mice. At day 28 mice were culled, shaved and imaged using the FX Pro. Livers, spleens and any other areas of tumour infiltration were harvested for analyses by flow cytometry.

All 3 mice receiving 5 x 10^6 cells developed disease which was readily detectable by imaging (Figure 4.1). At harvest, each mouse had visible liver lesions and 2 mice also had large ovarian tumours. The findings at harvest corresponded well with the images achieved by the FX Pro which were able to identify both diffuse and localised disease (Figure 4.1). 1 of the 3 mice receiving the lower dose of 10^6 cells also developed disease that was detectable by imaging and was found to have an ovarian tumour at harvest. Flow cytometry confirmed the presence of DSRed +ve

Figure 4.1: *In vivo* imaging identifies both diffuse and localised disease. By superimposing fluorescent images on white light images disease is localised via identification of the cellular DSRed fluorophore in malignant tissue. Diffuse bioluminescent disease (left panel, left mouse) and localised live lesions (left panel, central mouse, demonstrated by arrow) are clearly visible. At the time of harvest, large granulocytic lesions were often seen arising from the ovaries (right panel, ovarian mass indicated by arrow).

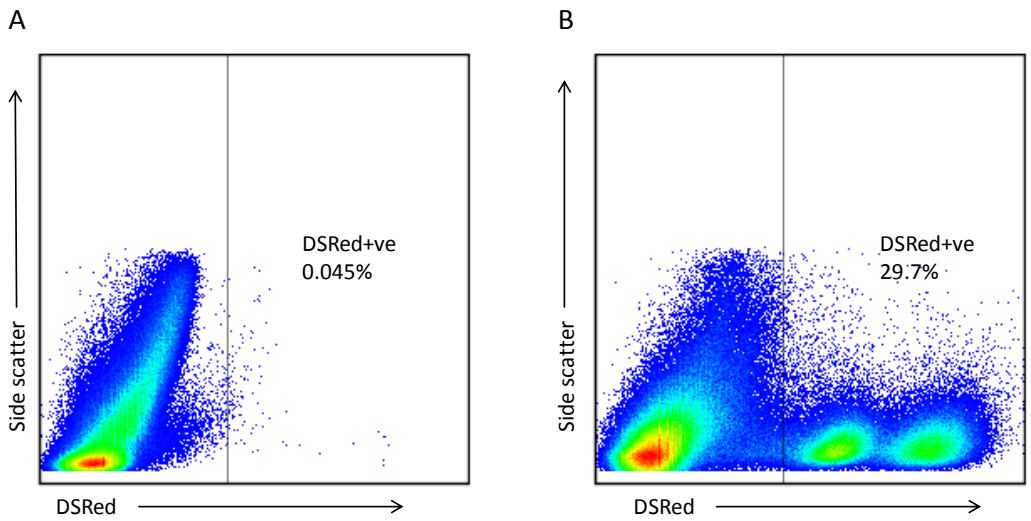


Figure 4.2: DSRed +ve granulocytic infiltration. Representative flow cytometric plots demonstrating DSRed +ve AML cells infiltrating the liver of an experimental mouse B) as compared to a control animal A).

granulocytic infiltration in the organs of affected mice, but not in the 2 mice receiving the lower dose of cells which were negative on imaging (Figure 4.2). It was therefore concluded that the higher dose of 5 x 10^6 cells IV resulted in consistent disease with no adverse effects.

4.2.2. AML fails to progress in mice lacking C3

There are several components of the complement system which might have a role in the progression of AML. In order to assess the over-reaching effect of a range of these components, the progression of AML in a fully complement deficient environment was first investigated. In order to do this genetically modified C3 knockout (C3-/-) mice were used. The C3-/- mice used in these experiments were 10 X back-crossed onto a B6 background. C3-/- mice lack the ability to produce the membrane attack complex and are therefore unable to lyse cells via complement-mediated lysis. They are also unable to produce the anaphylatoxins C3a and the opsonizing protein C3b and therefore lack the wide range of functions mediated by these proteins (Figure 4.3).

In order to determine whether any complement components below the level of C3 had an impact on progression of AML, 8 female age matched WT and C3-/- mice received 5 x 10^6 tumours cells IV on day 0. The development of disease was monitored using the FX Pro with fluorescent images being superimposed onto white light images in order to allow accurate disease localisation of the cellular DSRed fluorophore in malignant tissue. Mice were shaved and imaged under inhaled isofluorane anaesthetic weekly for 2 weeks, twice weekly for 3 weeks and then weekly until the end of the experiment. An imaging and health (IH) score which scored both imaging findings and health factors was used to allow consistent decisions to be made on experimental endpoints (Table 2.2). Mice were harvested when they reached a combined imaging and health (IH) score of 4.

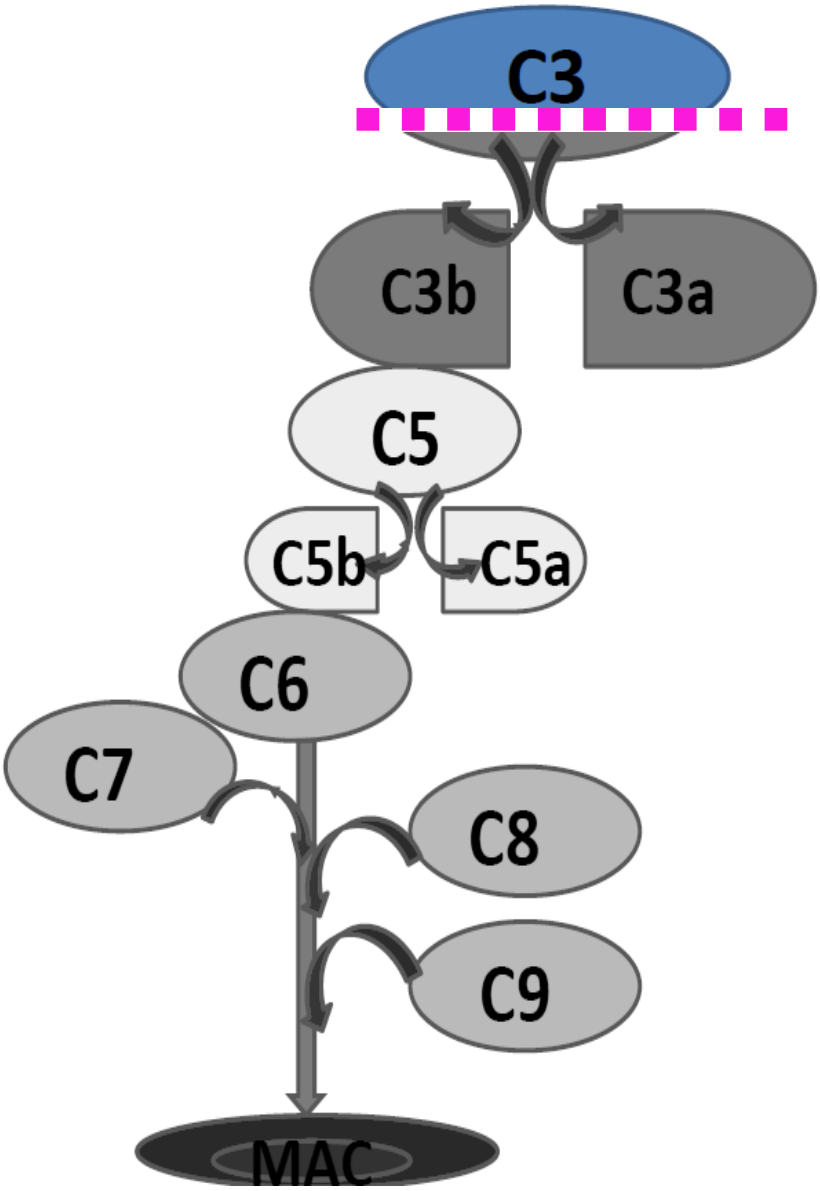


Figure 4.3: Phenotype of C3 deficient (C3-/-) mouse. C3-/- mice lack the ability to produce the membrane attack complex and are therefore unable to lyse target cells via complement-mediated lysis. They are also unable to produce the anaphylatoxin C3a and the opsonizing protein C3b and therefore lack the wide range of functions mediated through these proteins.

The development of leukaemia was readily detectable in shaved, anaesthetised mice using serial imaging with the FX Pro. Disease was detectable from day 15 in WT controls with all 8 mice being harvested between days 23 to 37.

No tumours were detected in the C3-/- mice, despite repeated imaging for a total of 77 days and full examination of all mice post mortem (Figures 4.4 and 4.5).

Over the course of 4 experiments a total of 32 C3-/- mice (24 female, 8 male) received IV C1498 at a dose of 5 x 10^6 cells that was lethal to age and sex matched WT mice (Figure 4.6). Most C3-/- mice (29/32) failed to develop detectable disease, P <0.0001***, median survival WT = 29.5 days, C3-/- = not reached. It was concluded that C3-/- mice, which lack the ability to form any component of the complement cascade, are resistant to progression of AML when injected IV with the murine AML cell line C1498.

4.2.3. T Cells are required to control growth of AML in C3-/- mice

Having demonstrated that AML fails to progress in a complement–deficient environment, how this protection was mediated was next explored. Complement has previously been shown to promote tumour progression via impingement on CD8+ T cell responses. Chapter 3 described a possible role for complement in impinging on both CD8+ T cell and NK mediated immune responses via complement-induced protection. Whether NK or CD8+ T cell depletion had any effect on progression of AML in a complement deficient vs sufficient environment was therefore next explored.

The anti-mouse-NK monoclonal antibody PK136 and the combination of two anti-mouse-CD8+ antibodies, YTS-169 and YTS-156, have been used widely by our group to deplete NK and CD8+ T cells in WT mice *in vivo.* Their efficacy has not as yet been explored in a complement deficient environment. As the function of some antibodies depends on the presence of complement, the ability to deplete NK or CD8+ T cells in the absence of complement was assessed.

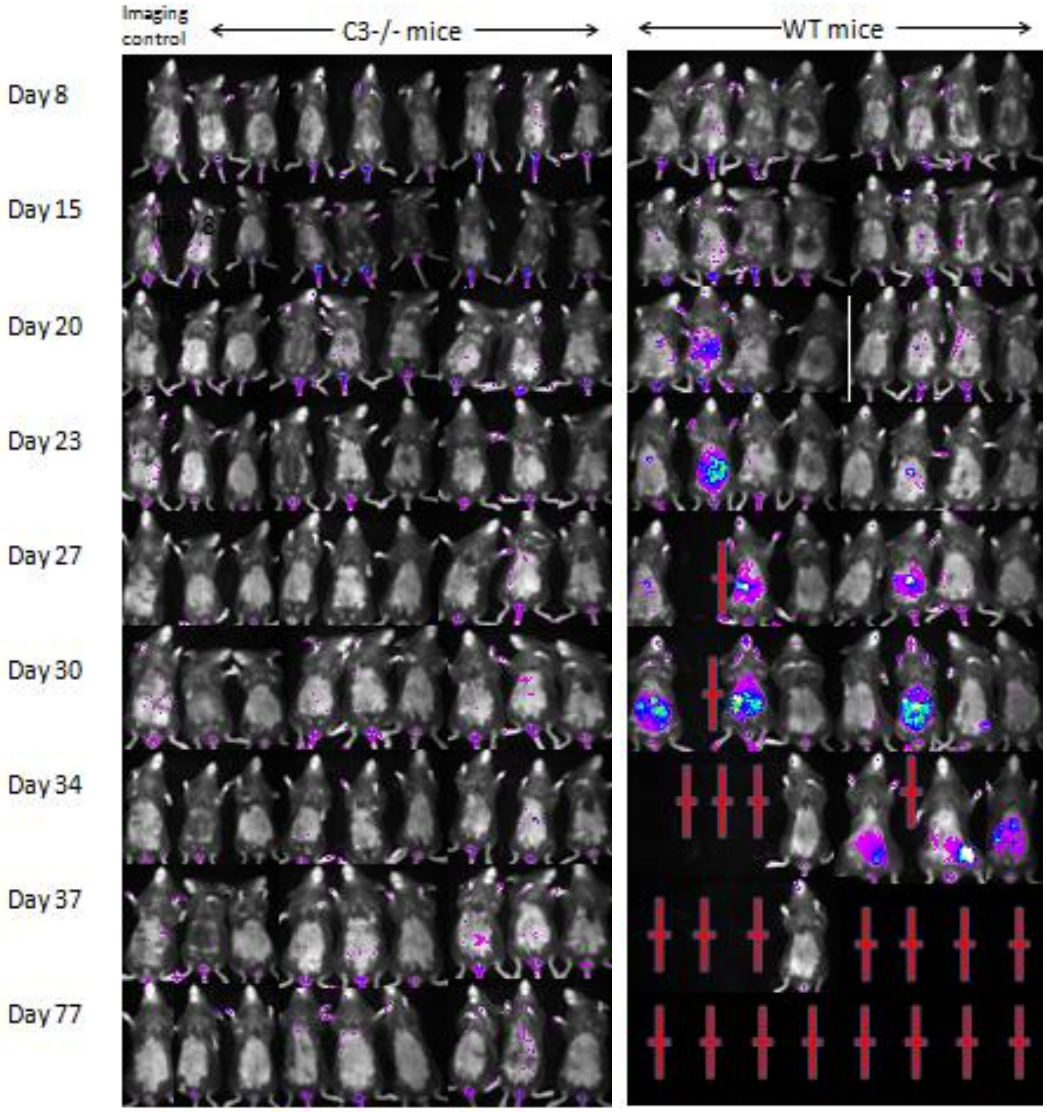


Figure 4.4: Serial *in vivo* imaging of DSRed+ve AML. Serial imaging of wild type (WT) and C3-/- mice demonstrated detectable disease in WT mice resulting in the animals being killed by day 37. C3-/- mice were imaged for a further 40 days. Over the course of the 11 week experiment, no C3-/- mice developed AML as detected by either serial imaging or post mortem examination at the end of the experiment.

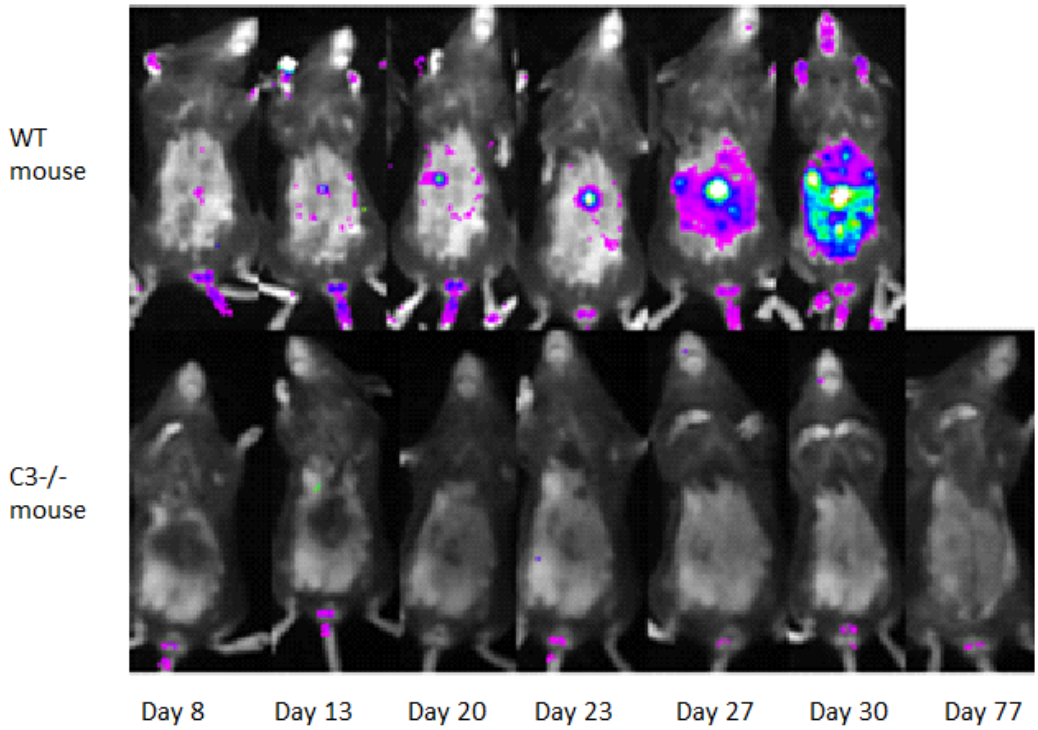


Figure 4.5: *In vivo* imaging showing disease progression. Images demonstrate disease progression in a representative WT mouse over a period of 30 days. In contrast, C3-/- mice failed to develop detectable disease.

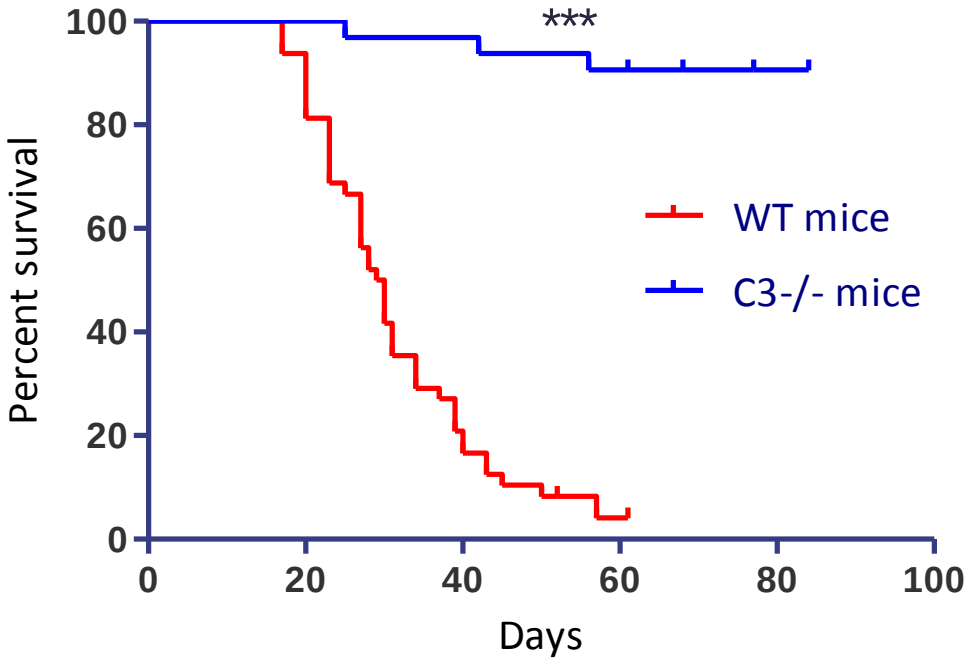


Figure 4.6: Survival of C3-/- vs WT mice. 5 x 10^6 C1498 cells injected IV into a total of 48 WT and 32 C3-/- mice. Kaplan Meier survival curve; P = <0.0001*** (Log-rank (Mantel-cox) test). Median survival WT = 29.5 days, C3-/- = not reached.

In order to do this 6-9 week old female C3-/- mice were treated with either anti-NK or anti-CD8 antibodies. The anti-NK antibody PK136 was used at a dose of 0.5mg IP (2 mice). Two anti-CD8 antibodies (YTS-169 and YTS-156) were used in combination, each at a dose of 100µg IP (2 mice). Dosing was repeated 3 days later. On day five these mice along with a control mouse were harvested and their spleens analysed for the presence of NK and CD8+ T cells.

Results demonstrated that the combined use of two CD8 depleting antibodies, YTS-169 and YTS-156, depleted CD8+ T cells in a C3-/- mice (Figure 4.7). Although not complete, the 85-90% CD8+ depletion observed suggested that the antibodies were able to deplete the majority of CD8+ T cells even in the absence of complement. We therefore went on to use these antibodies to explore the effect of CD8+ T cell depletion on tumour progression in C3-/- mice.

Contrary to this, the NK depleting antibody PK136, did not appear to deplete NK cells in C3 deficient mice (Figure 4.8), implying that depletion of NK cells by PK136 is complement-dependent, a finding supported by other studies(Janelle, Langlois et al. 2014). I was therefore unable to further explore the impact of NK cells on progression of AML in C3-/- mice.

In addition to CD8+ T cell depletion we also explored the effect of CD4+ T cell depletion on the progression of AML in complement sufficient vs deficient environment. The combination of 2 CD4 depleting antibodies YTS-191 and YTA-3, which work via a similar mechanism to the CD8 depleting antibodies YTS-169 and YTS 156, were used in these studies.

Groups of eight 7-13 week old C3-/- or age matched WT mice were injected on day -3, day -1 and then fortnightly with a total of 200µg of either CD8 depleting (100µg of each YTS-169+ YTS-156), CD4 depleting (100 µg of each YTS-191 and YTA-3) or control (GL113) antibodies. On day 0 all mice received 5 x 10^6 IV C1498FFDsR cells.

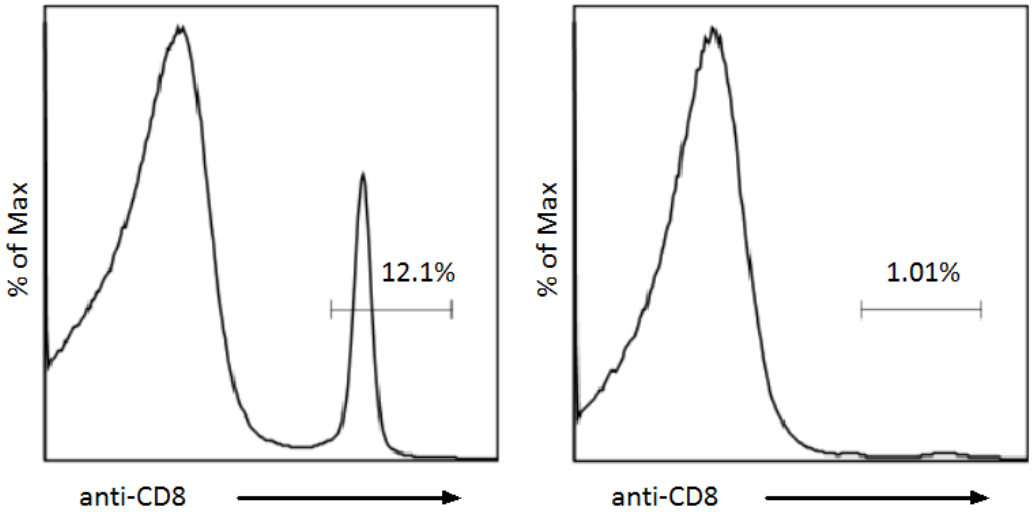


Figure 4.7: CD8-depletion in representative C3-/- mice. Two CD8-specific depleting antibodies, YTS-169 and YTS-156, were used in combination to deplete CD8+ T cells in two C3-/- mice. The antibodies were each administered at a dose of 100µg IP, this dose was repeated 3 days later. On day five mice were harvested along with an untreated control. Following red cell lysis, splenocytes were stained with CD8-specific antibodies along with live/dead aqua and analysed by flow cytometry for the presence of live CD8+ cells.

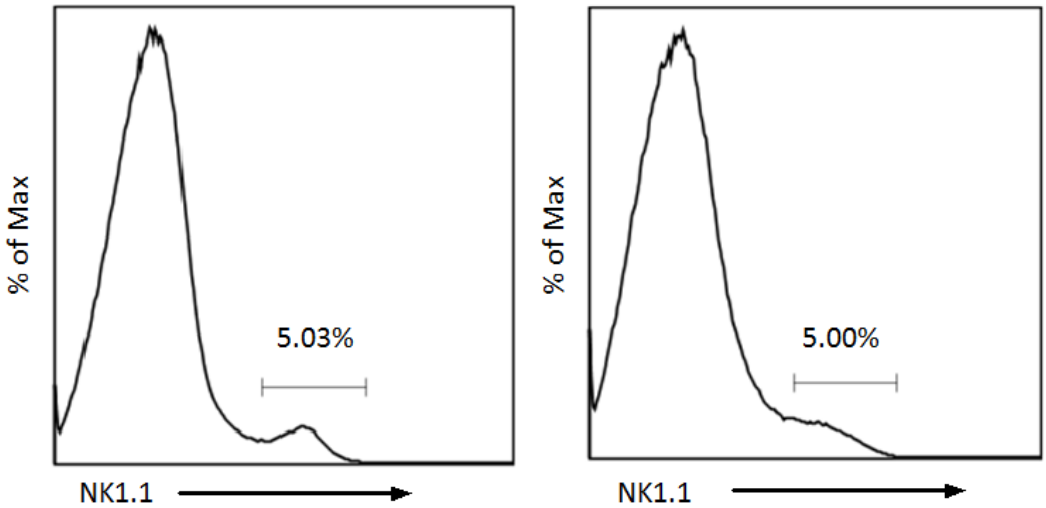


Figure 4.8: Attempted NK depletion in a representative C3-/- mouse. The NK depleting antibody PK136 was administered 0.5mg IP (2 mice) with repeat dosing 3 days later. Mice were harvested along with an untreated control on day 5. Following red cell lysis, splenocytes were stained with a FITC conjugated anti NK1.1 antibody along with live/dead aqua and analysed by flow cytometry for the presence of live NK cells. There was no evidence of successful NK depletion in the C3-/- mice suggesting that the PK136 antibody depletes NK cells via a complement mediated mechanism.

Mice were imaged twice weekly from day 7 (Figure 4.9) and harvested when a combined IH score of 4 was reached.

Untreated WT mice began developing disease from day 20 with 5 being harvested between days 20 to 31. Two further untreated WT mice were harvested on day 40 and there was one long-term survivor. CD8-depleted WT mice had a more aggressive phenotype than controls with lesions developing from day 14 and all mice being harvested by day 17. P <0.0001***, median survival: WT = 31 days, CD8-depleted = 18 days. CD4-depleted WT mice also showed the first signs of disease on day 14 with 6 mice being harvested between days 17 and 25. One CD4-depleted WT mouse developed diffuse disease by day 54 and there was one long-term survivor. There was no statistically significant difference in survival observed between CD4+ T cell depleted and WT mice. P = 0.3588 (ns), median survival: WT 31 days, CD4-depleted 25 days (Figure 4.10).

As previously shown, untreated C3-/- mice exhibited a highly significant reduction in disease progression compared to untreated WT mice. In contrast, the CD8 depleted C3-/- mice began developing detectable lesions on day 14, with 6 mice being harvested between days 17 and 28. The remaining 2 mice were long-term survivors. This resulted in a highly significant survival advantage being observed in untreated vs CD8-depleted C3-/- mice, P = 0.0036** (Log-rank (Mantel-cox) test). Median survival: untreated C3-/- = not reached, CD8-depleted C3-/- = 20 days. CD4-depleted C3-/- mice began developing lesions from day 20 with 6 mice being harvested between days 20 and 31. There were again, 2 long-term survivors. The difference between CD4-depleted and untreated C3-/- mice just reached statistical significance, P = 0.049* (Log-rank (Mantel-cox) test). Median survival: CD4-depleted C3-/- = 28 days, C3-/- control = not reached (Figure 4.11).

Despite disease developing in the CD8-depleted C3-/- mice, this was significantly less aggressive than disease seen in CD8-depleted WT mice P = 0.0014**. Median survival: CD8-depleted WT = 18 days, CD8-depleted C3-/- = 20 days. Disease progression in CD4-depleted C3-/- mice was comparable to that seen in their CD4-

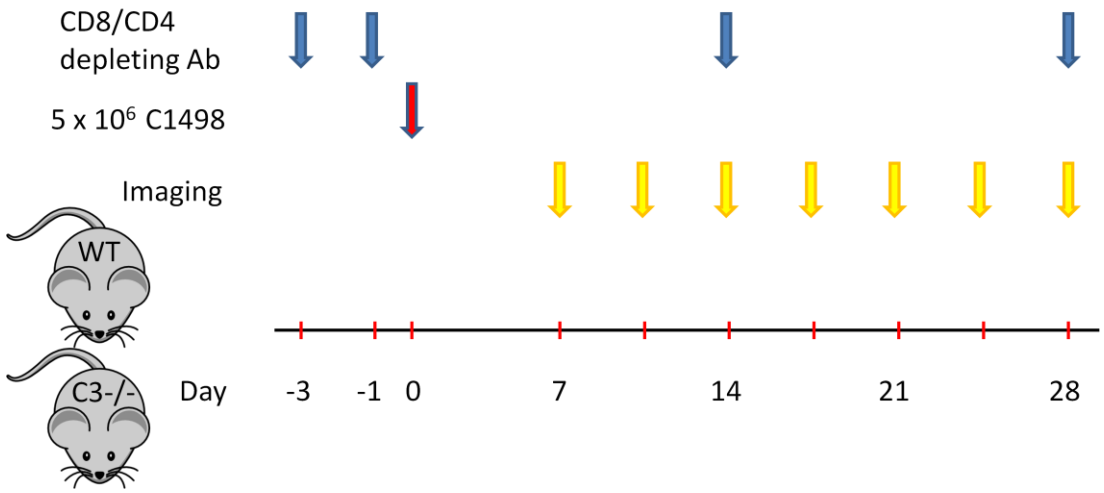
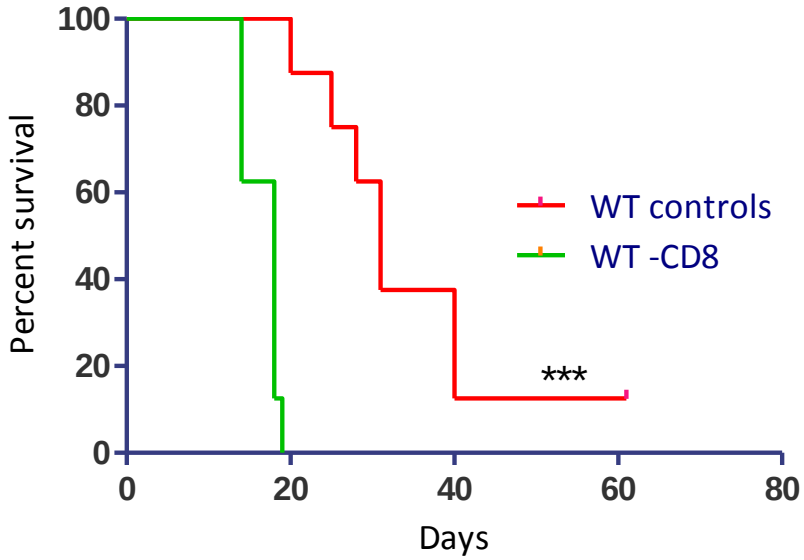


Figure 4.9: Protocol for *in vivo* T cell depletion studies. Groups of 8 age and sex-matched B6 or C3-/- mice were treated with CD8-depleting (YTS-169 and YTS-156 100µg of each), CD4-depleting (YTS-191 and YTA-3 100µg of each) or control (GL113) antibodies. In each group, a combined dose of 200µg was administered IP on day -3, day -1 and then fortnightly. Mice received 5 x 10^6 C1498 cells IV on day 0 and were then imaged twice weekly from day 7.

A.



B.

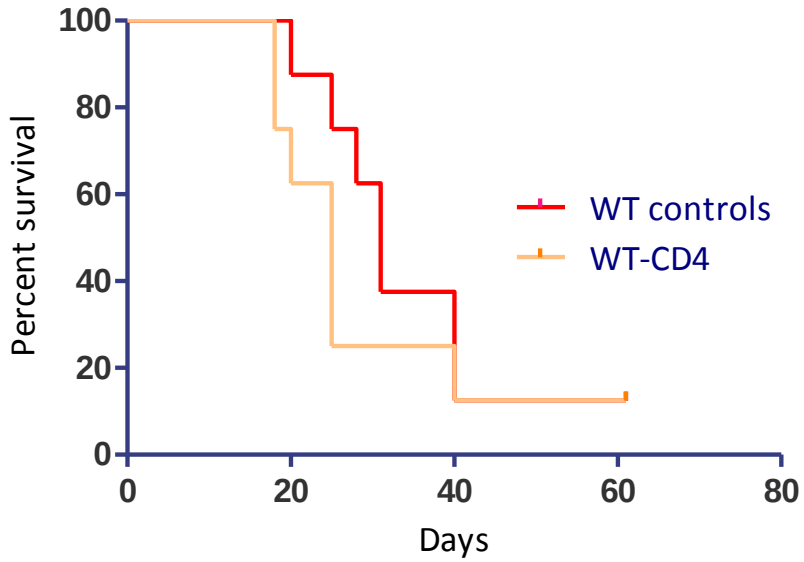
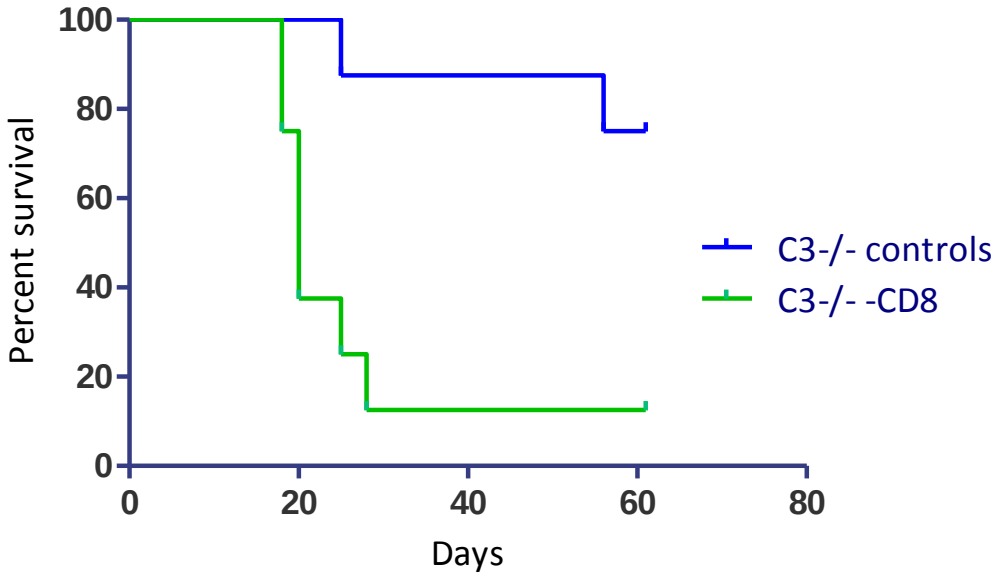


Figure 4.10: Survival of WT mice depleted of CD8+ or CD4+ cells. 5 x 10^6 C1498 cells injected IV into 8 CD8-depleted and 8 CD4-depleted WT mice along with 8 age and sex matched controls. Kaplan Meier survival curve; A) +/- CD8-depleted P <0.0001*** (Log-rank (Mantel-cox) test). Median survival WT = 31 days, CD8-depleted = 18 days. B) +/- CD4-depleted P = 0.3588 (ns). Median survival WT = 31 days CD4-depleted = 25 days.

A.



B.

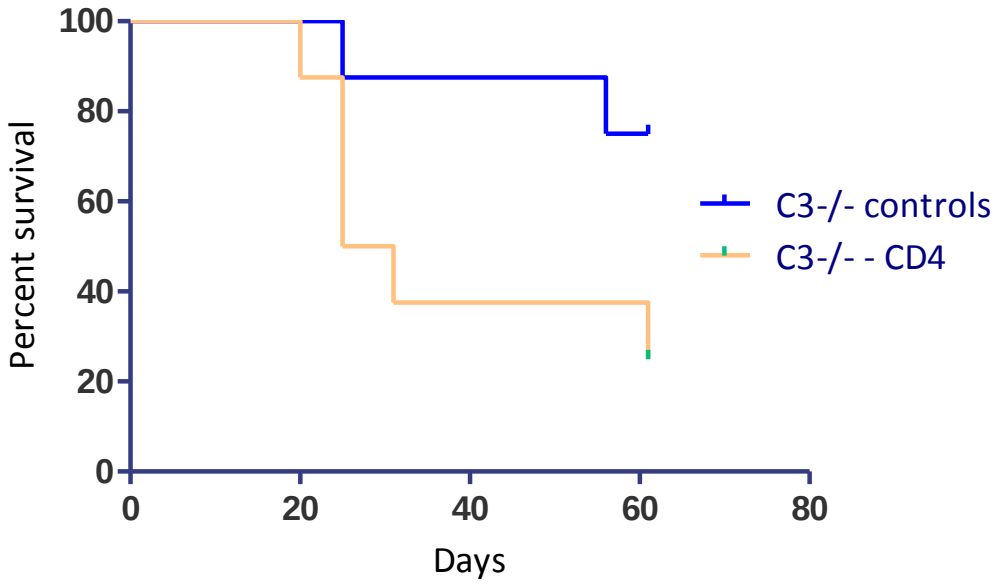


Figure 4.11: Survival of C3-/- mice depleted of CD8+ or CD4+ cells. 5 x 10^6 C1498 cells injected IV into 8 CD8 depleted and 8 CD4 depleted C3-/- mice along with 8 age and sex matched controls. Kaplan Meier survival curve; A) +/- CD8+ T cell depletion P = 0.0036** (Log-rank (Mantel-cox) test). Median survival C3-/- = not reached, CD8 depleted = 20 days. B) +/- CD4+ T cell depletion P = 0.049*. Median survival CD4 depleted C3-/- = 28 days.

depleted B6 counterparts leading to no significant difference being demonstrated between the two groups P = 0.2804 (ns). Median survival: CD4-depleted WT = 25 days, CD4-depleted C3-/- = 28 days (Figure 4.12). Table 4.2 provides an overview of the statistically significant findings.

Experimental Group		Experimental Group	P-value	Significant?
C3-/-	vs	**WT**	**0.0098**	Yes **
C3-/-	vs	**C3-/- - CD8**	**0.0036**	Yes **
C3-/-	vs	**C3-/- - CD4**	**0.049**	Yes *
C3-/- - CD8	vs	**WT - CD8**	**0.0014**	Yes **
C3-/- - CD4	vs	**WT - CD4**	**0.2804**	No
WT	vs	**WT - CD8**	**<0.0001**	Yes ***
WT	vs	**WT - CD4**	**0.3588**	No

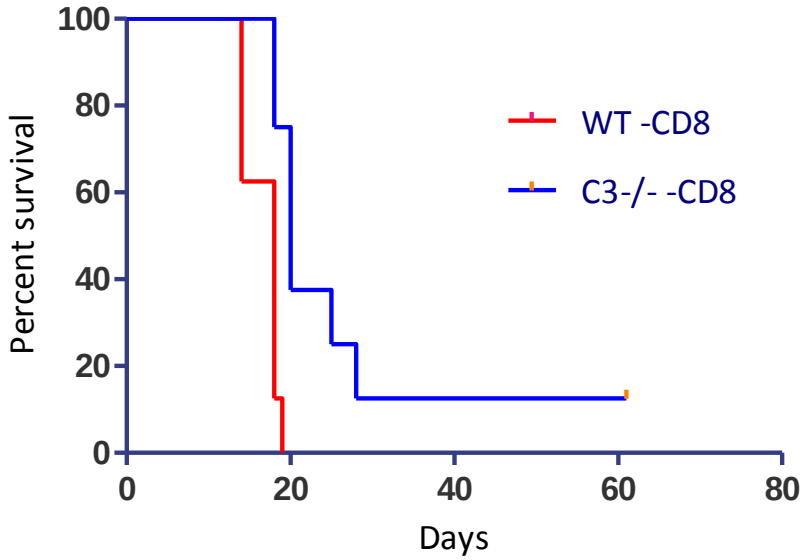
Table 4.2: Summary of CD4/CD8-depletion studies. Log-rank (Mantel-Cox) tests were used to assess statistical significance between WT and C3-/- mice +/- CD8 or CD4 depleting antibodies.

In conclusion, CD8- (and to a lesser extent CD4-) depletion resulted in C3-/- mice consistently developing AML. This suggests that complement deficiency protects against the progression of AML via a mechanism mediated by CD8+ (and/or CD4+) T cells.

4.2.3. C1498 cells produce no detectable C3 protein

Several malignant cell lines have been shown to produce C3 (Kitano and Kitamura 1993; Legoedec, Gasque et al. 1995; Andoh, Fujiyama et al. 1998). It is therefore possible that the C1498 AML cell line produced C3 which would be immunogenic in C3-/- mice since the mice would not have encountered C3 during thymic selection. This could result in immune mediated clearance of the C1498 cells through T cell recognition of MHC / C3 peptide complexes. Evidence of C3 production by C1498 cells was therefore investigated.

A.



B.

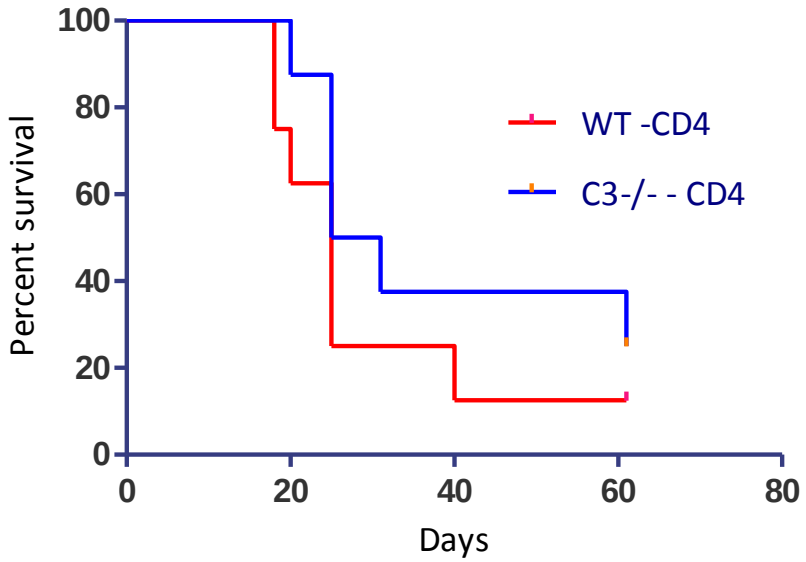


Figure 4.12: Survival of C3-/- vs WT mice depleted of CD8+ or CD4+ T cells. 5 x 10^6 C1498 cells injected IV into 8 CD8 depleted and 8 CD4 depleted WT and C3-/- mice. Kaplan Meier survival curve; A) CD8+ T cell depletion in C3-/- vs WT mice P = 0.0014** (Log-rank (Mantel-cox) test). Median survival CD8 depleted WT = 18 days, CD8 depleted C3-/- = 20 days. B) CD4+ T cell depletion in C3-/- vs WT mice P = 0.2804 (ns). Median survival CD4 depleted WT 25 days CD4 depleted C3-/- = 28 days.

In order to determine whether C1498 cells were producing C3 *in vitro* a C1498 cell lysate along with a 20X concentrated C1498 supernatant were tested for the presence of C3 by Western Blot. Mouse serum and concentrated mouse C3 were used as positive controls and C3-/- mouse serum was used as a negative control. There was no evidence that C1498 cells produce C3 *in vitro (*Figure 4.13).

The ability for C1498 cells to produce C3 *in vivo* was then assessed by immunohistochemistry. Discrete C1498 tumours, which had developed above the ovaries of C3-/- mice were stained for C3 deposition by malignant cells *in vivo*. As only 1 C3-/- mouse had developed solid tumours, ovarian tumours from CD8-depleted C3-/- mice were also used allowing multiple tumours taken from WT and C3-/- to be assessed. No evidence of C3 production by C1498 tumours *in vivo* was observed (Figure 4.14).

4.2.4: C3-/- mice do not mount a better CD8+ T cell response against C1498 leukaemia cells

AML fails to progress in CD8+ T cell replete C3-/- mice however when CD8+ T cells are depleted C3-/- mice develop AML. It was therefore postulated that the CD8+ T cell response against AML was more robust in a complement deficient vs sufficient environment. In order to investigate this, both WT and C3-/- mice were vaccinated with irradiated AML cells. Irradiated cells were used as previous experiments suggest that the kinetics of AML progression following injection of live cells is unlikely to be comparable between WT and C3-/- mice. Irradiating cells removed any disparities introduced by variable growth kinetics thus delivering a more comparable dose/ stimuli in WT vs C3-/- mice.

Splenocytes from vaccinated/ unvaccinated WT vs C3-/- mice were then assessed for IFNγ release in response to irradiated C1498 in an ELISpot assay. Full details are provided in Chapter 2 but in brief, 4 WT and 5 C3-/- mice were vaccinated SC with 5 x 10^7 irradiated C1498 cells. Vaccination was repeated after 4-6 weeks with

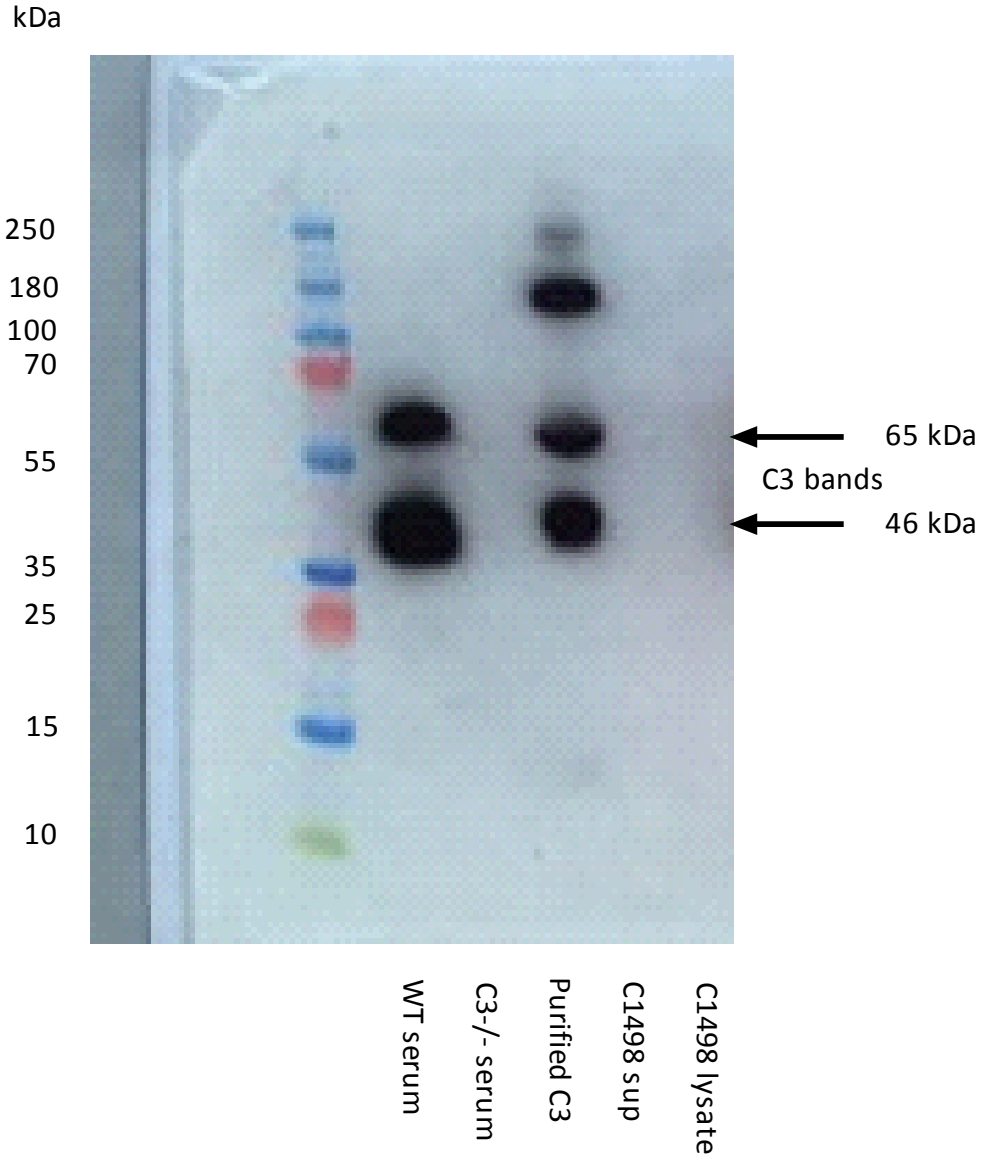


Figure 4.13: Immunoblot of C3. HRP conjugated goat anti-mouse C3 antibodies were used at 0.1µg/ml in order to detect C3. Two positive control samples (WT mouse serum and purified mouse C3) along with one negative control (C3-/- mouse serum) were included on the blot. Two bands characteristic of C3 staining were observed in both positive controls (arrows, β-chain = 65kDa, α-chain fragments approx 46kDa). A C1498 cell lysate along with 20 x concentrated supernatant from confluent C1498 cells were tested for expression of C3. No C3 was detected.

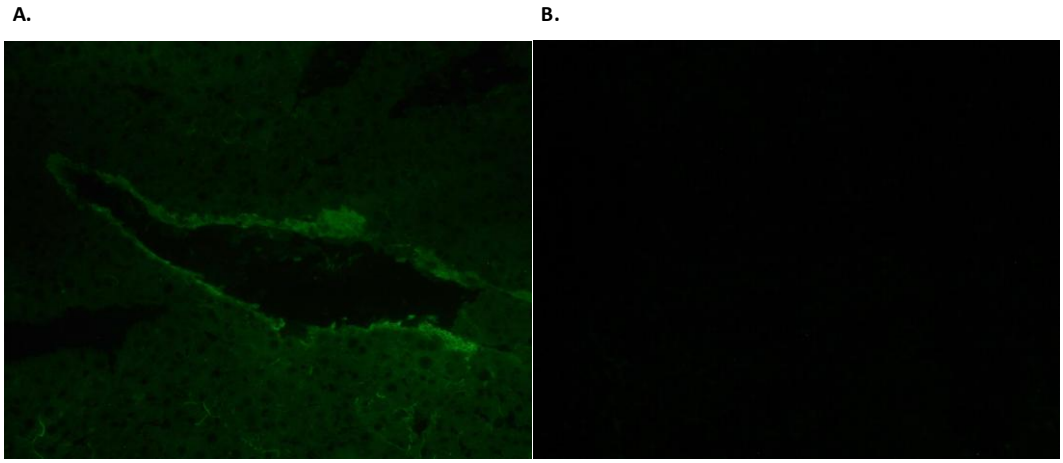


Figure 4.14: C3 staining on a liver from a WT mouse and C1498 ovarian tumour from a C3-/- mouse. C3 deposition was assessed using a two step staining protocol as detailed in Chapter 2. A rat anti-C3 monoclonal antibody (11H9) was used with a 488 anti-rat secondary detection antibody alongside an isotype control antibody (negative, not shown). Multiple tumours taken from WT and C3-/- mice were assessed. A. C3 deposition around a venule in a liver taken from a tumour bearing WT mouse (arrow). B. Cross section through a solid ovarian tumour harvested from a C3-/- mouse (CD8 depleted) revealed no evidence of C3 deposition.

vaccinated mice being harvested 7 days later along with 3 WT and 3 C3-/- unvaccinated controls. Splenocytes were then assessed for their IFNγ response to irradiated C1498 at a range of effector: target ratios. Results demonstrated that all vaccinated mice responded to the cell line in the ELISpot assay. The response observed in C3-/- mice was no better than seen in WT mice (Figure 4.15).

4.2.5: The impact of components of the complement pathway on progression of AML

Thus far it has been demonstrated that C3-/- mice are protected against progression of C1498 leukaemia. This appears to be mediated through CD8+ T cells although the exact mechanism remains unclear. As C3-/- mice lack several potentially relevant components of the complement cascade including C3a, C3b and MAC. Which components of the complement system were key to disease progression was therefore investigated (Figure 4.16).

The three main mechanisms identified as having a potential role in the progression of AML were:

i) Anaphylatoxins, C5a and C3a
ii) Complement-induced protection
iii) Cell adherence and migration

(Figure 4.17)

In order to systematically investigate the role of complement in the progression of AML, genetically complement-deficient mice, or mice administered with complement inhibitors, to block or inhibit complement activation respectively were used.

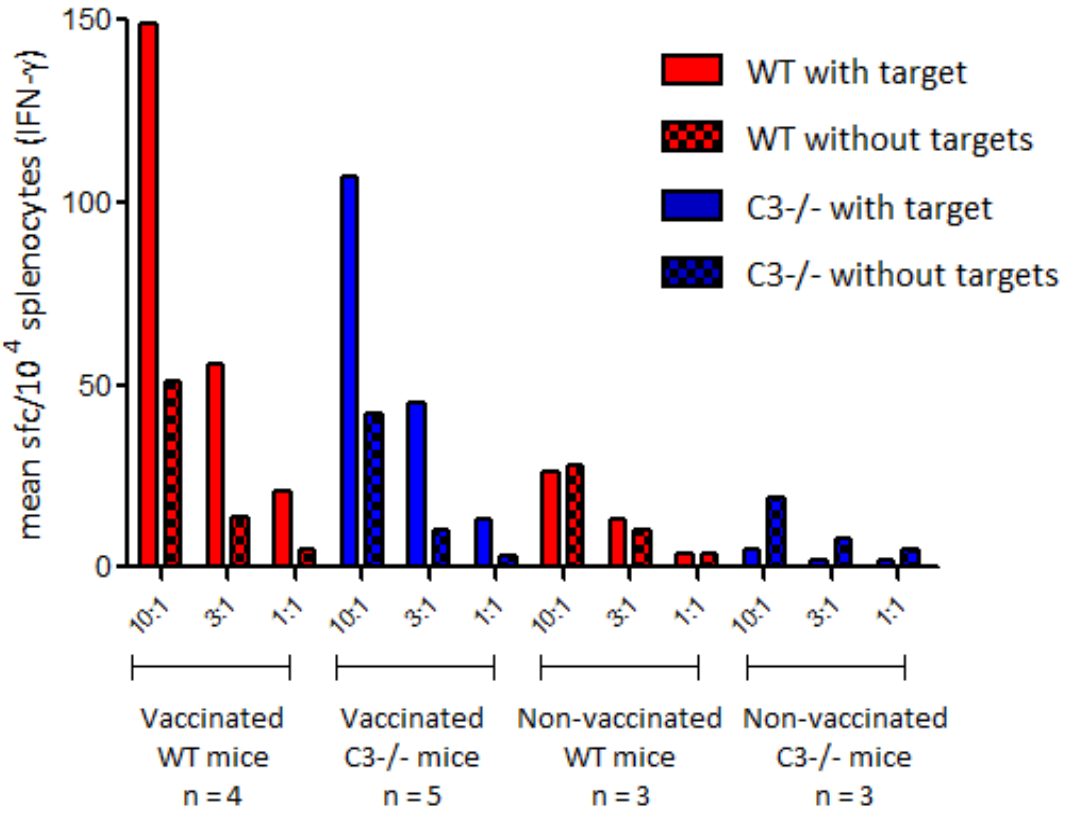


Figure 4.15: Graph demonstrating ELISpot responses to vaccination with irradiated C1498 cells in WT vs C3-/-mice. WT and C3-/- mice were vaccinated with 5 x 10^7 irradiated C1498 cells. This was repeated 4 weeks later. 7 days later animals were harvested, along with unvaccinated control animals. Splenocytes were tested for their response to irradiated C1498 cells as targets at a range of E:T ratios. IFN-γ secreting cells were analysed and a mean responses from 4 WT, 5 C3-/- vaccinated animals and 3 non-vaccinated WT and C3-/- controls are shown.

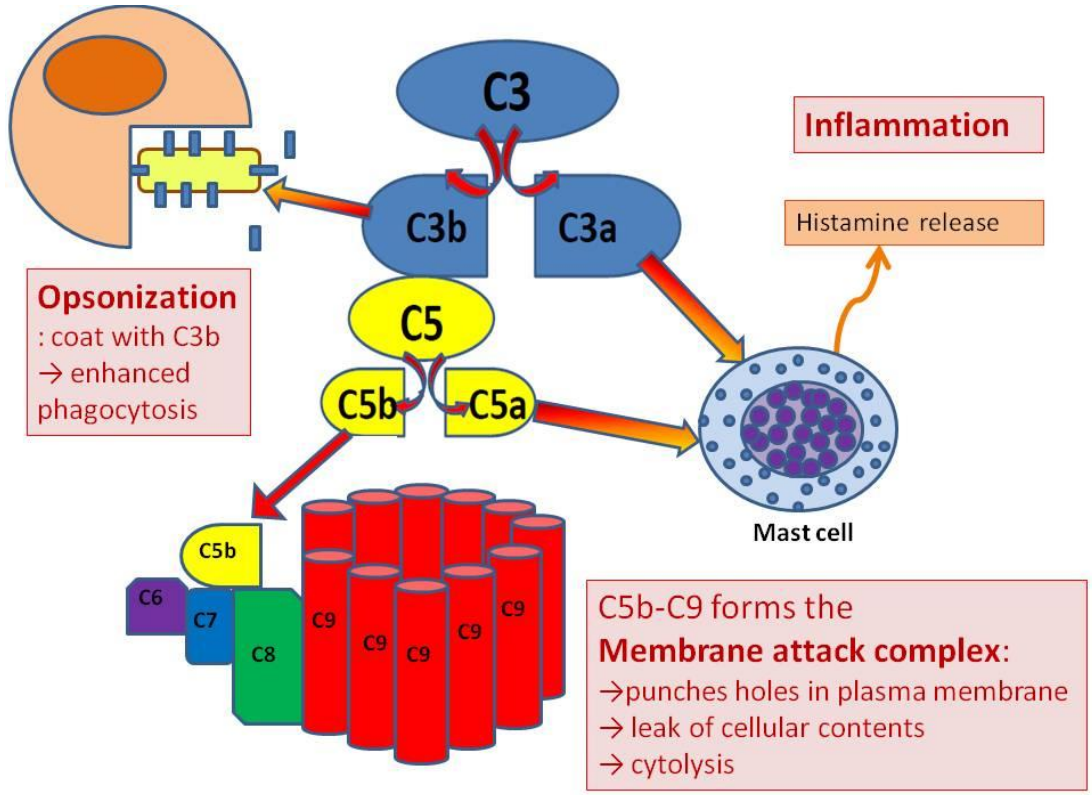


Figure 4.16: Schematic of the complement cascade demonstrating areas to be explored in an *in vivo* model of AML. C3, C3a receptor and C6 knockout mice were utilised along with anti-C5 blocking antibodies, C5a receptor antagonist and C6 antisense oligonucleotide.

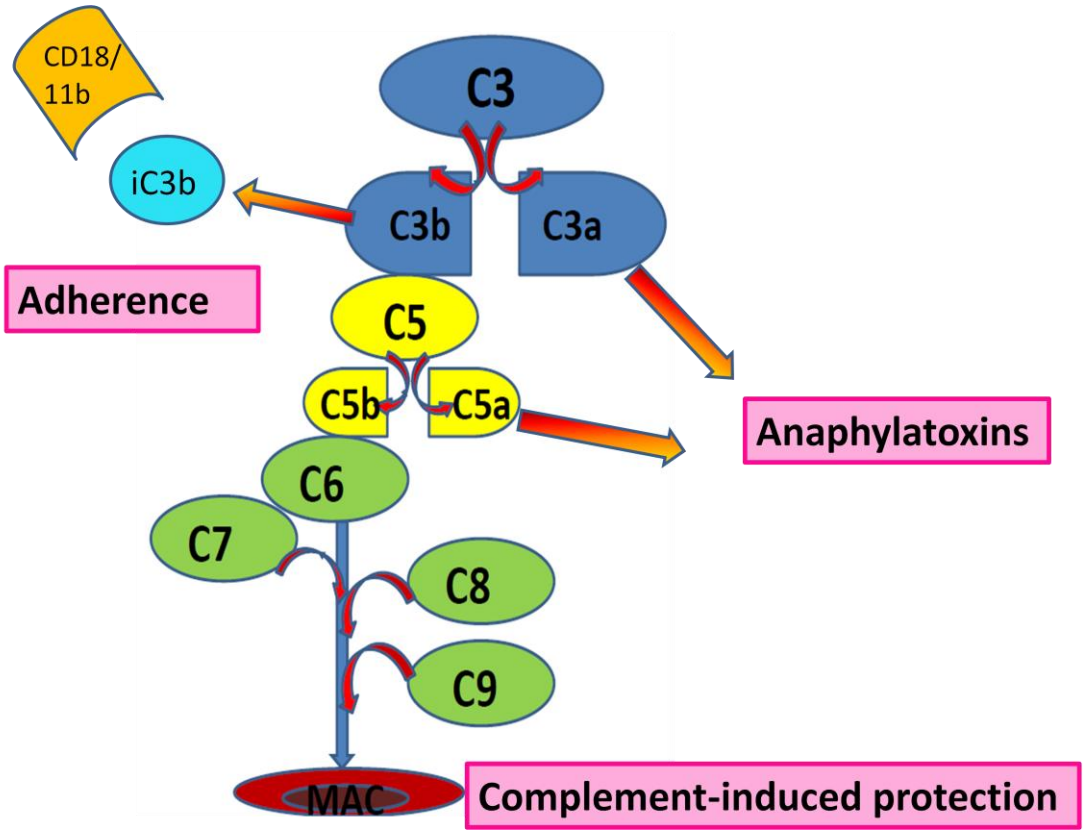


Figure 4.17: Components of the complement cascade that might contribute to the protective phenotype. Three mechanisms were identified through which complement could be mediating the progression of AML i) anaphlylatoxins ii) complement-induced protection and iii) adherence and cell migration.

4.2.5.1. The role of the anaphylatoxins C5a and C3a

C5a Anaphylatoxin

Investigating the role of the complement anaphylatoxins, particularly C5a, was the most obvious starting point. C5a has been shown to create a favourable environment for tumour growth by modulating the anticancer immune response via suppression of the anti-tumour CD8+ T cell response by myeloid derived suppressor cells (MDSCs) (Markiewski, DeAngelis et al. 2008). Other studies have also implicated C5a in promoting tumour growth via both similar and distinct mechanisms such as promoting the activation of Tregs, or increasing tumour infiltration with macrophages or monocytes (Caso, Silvera et al. 2010; Corrales, Ajona et al. 2012; Gunn, Ding et al. 2012; Nunez-Cruz, Gimotty et al. 2012; Vadrevu, Chintala et al. 2014; Imamura, Yamamoto-Ibusuki et al. 2015). These studies suggest the immunomodulatory functions of C5a are highly context dependent.

It was hypothesised that blocking complement activation at the level of C5, which would both prevent the production of MAC and the anaphylatoxin C5a, would impact on progression of C1498 AML. The anti-mouse C5 antibody (anti-C5 mAb), BB5.1, was used (Figure 4.18).

The presence of endotoxin can have major effects on numerous immune functions (reviewed in (Heine, Rietschel et al. 2001)). Endotoxin-low anti-C5 mAb was therefore generated for use in these studies (see Chapter 2). The efficacy of the Ab was then assessed by first testing its ability to inhibit complement-mediated lysis *in vitro* (Figure 4.19). A dose of 3.125µg/ml anti-C5 mAb completely inhibited complement-mediated lysis of antibody-coated rabbit erythrocytes by 4% mouse serum *in vitro*.

Based on a review of published literature, a dose of 1mg IP was then assessed *in vivo.* Anti-C5 mAb was administered via an IP injection twice weekly beginning on day -1 (day 0 being the day of tumour cell injection). An isotype matched control

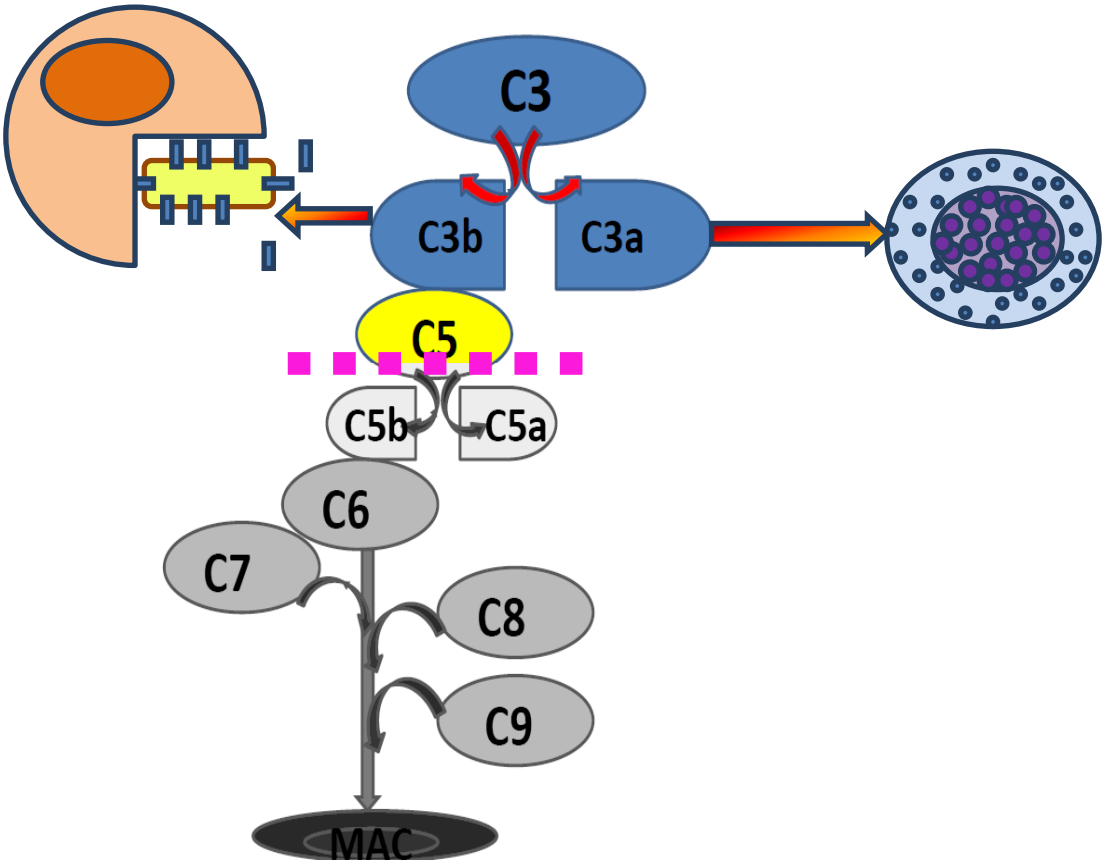


Figure 4.18: Phenotype achieved by the anti-C5 mAb. Mice treated with the anti-C5 mAb were unable to form MAC hence lacked any ability for complement mediated lysis. They are also unable to make the main complement anaphylatoxin C5a but retain the ability to produce both the anaphylatoxin C3a and the opsonising protein C3b.

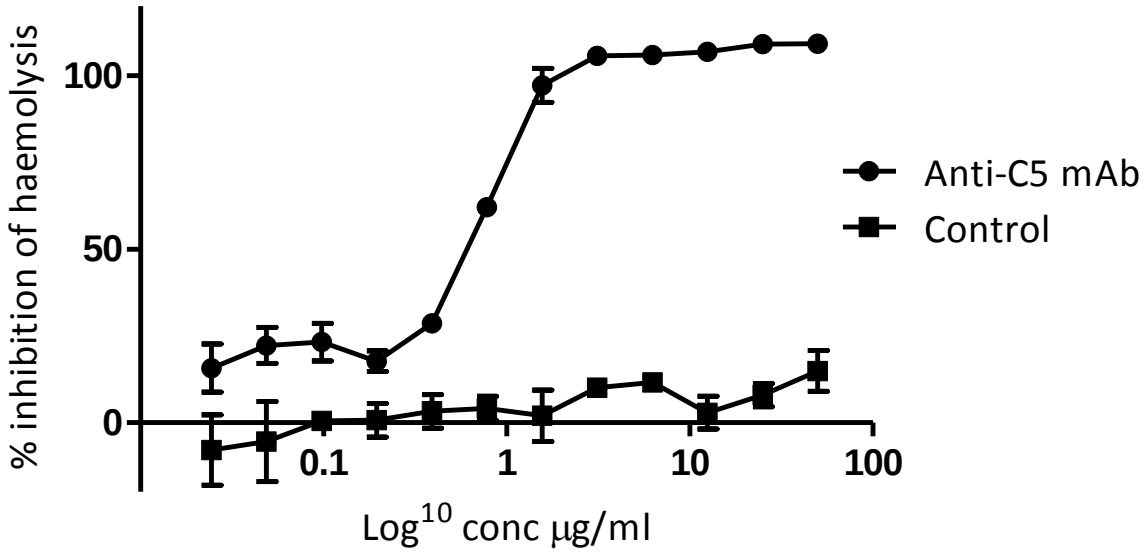


Figure 4.19: *In vitro* inhibition of complement by anti- C5 mAb. Rabbit erythrocytes were sensitised by incubation with a mouse anti-rabbit erythrocyte antibody. They were then treated with 4% normal mouse serum pre-incubated with a range of titres of anti-C5 mAb or the control antibody D1.3. Haemoglobin release was measured and expressed relative to 100% control (erythrocytes lysed with H_2O).

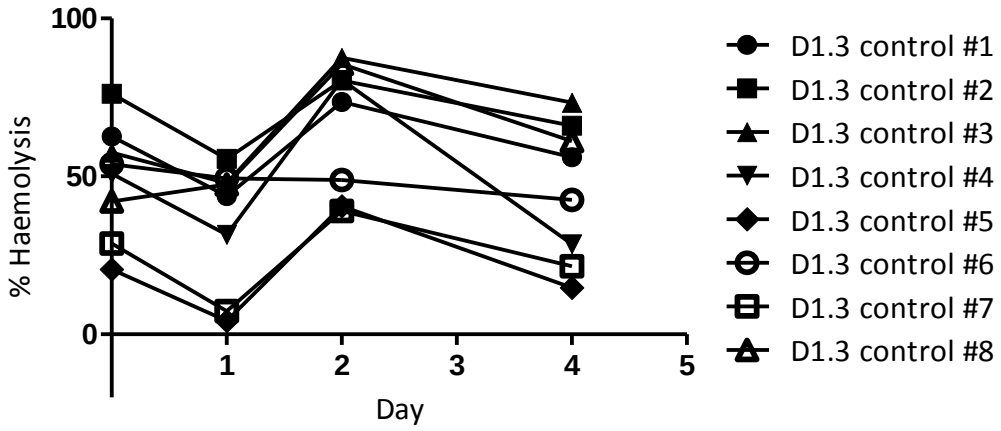
antibody (D1.3) was used at an equivalent dose in age- and sex-matched control mice. Blood was taken prior to the 1st injection and again on days 1, 2 and 4, such that adequate complement depletion could be confirmed up until the next dose of antibody was due. Complete inhibition of complement haemolytic activity was achieved at all time points in all mice treated with anti-C5 mAb (Figure 4.20). The dose of 1mg IP twice weekly was therefore taken forward into subsequent studies.

Groups of 8 age- and sex-matched WT mice were treated with either 1mg IP anti-C5 mAb or control antibody from the day prior to administration of 5 x 10^6 C1498 cells IV;subsequent maintenance doses of 1mg were administered twice weekly. *In vivo* imaging was performed twice weekly from day 7. This revealed equivalent disease development in treated and control mice P = 0.8270, median survival WT = 29.5 days, WT + anti-C5 mAb = 35.5 days (Figure 4.21).

There are three explanations for this result i) C5a has no effect on the progression of AML in this model ii) the method of C5a neutralisation was flawed or iii) additional effects of C5-depletion were affecting the results. It is possible that despite confirming a complete suppression of the haemolytic capacity of the complement system in plasma, the effect of the C5 depleting antibody was incomplete at a tissue level. C5a is produced at a local level and has important roles in mediating both $CD4^+$ and $CD8^+$ T cell responses (Lalli, Strainic et al. 2008; Strainic, Liu et al. 2008). In addition to preventing the production of C5a, anti-C5 mAb also inhibits the production of MAC. It is therefore possible that the absence of MAC was impinging on any protective effect conferred by the depletion of C5a (experiments described later in this Chapter).

Other studies investigating the role of C5a in malignancy have used the Cyclic hexapeptide antagonist AcF-[OPdChaWR] also known as PMX-53 in order to specifically block the main C5a receptor (Markiewski, DeAngelis et al. 2008; Corrales, Ajona et al. 2012; Vadrevu, Chintala et al. 2014). The independent role of the C5a-C5aR signalling axis was explored using this alternative reagent, which was kindly provided by Professor Trent Woodruff (Figure 4.22).

A.



B.

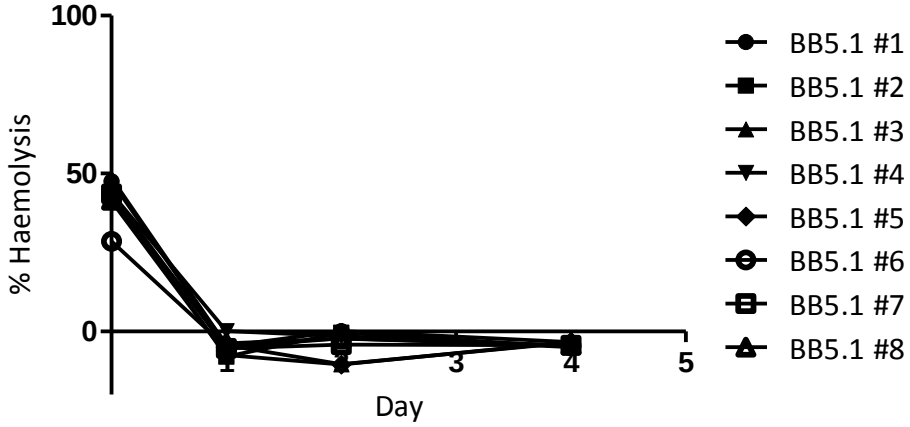


Figure 4.20: In *vivo* inhibition of complement. 8 mice had blood taken prior to administration of either 1mg IP of control antibody, D1.3 (A) or the anti-C5 mAb (B). Further blood samples were then taken on day 1 and 2 post dose and again on day 4, before a 2nd dose of antibody was due. Serum haemolytic ability was assessed using rabbit erythrocytes sensitised using mouse anti-rabbit erythrocyte antibody. Lysis was measured relative to 100% control erythrocytes lysed with H_2O.

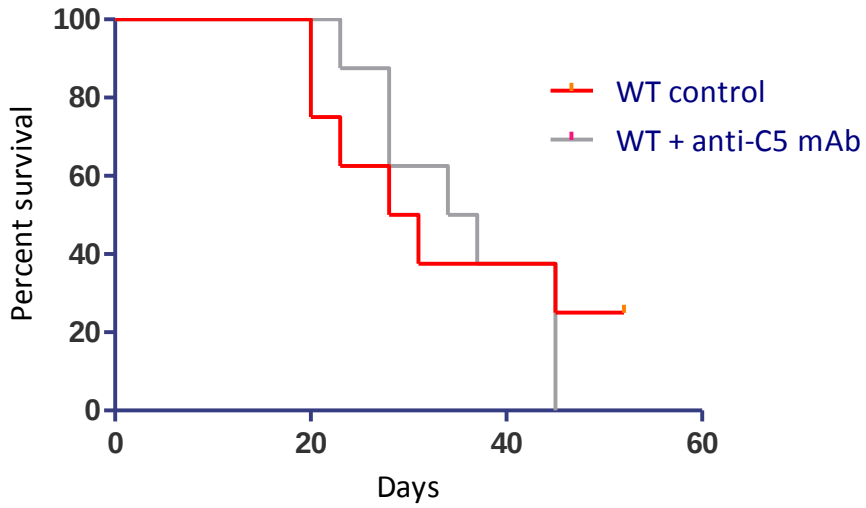


Figure 4.21: Survival of WT mice treated with anti-C5 mAb. 5 x 10^6 C1498 cells injected IV into a total of 8 WT mice treated with anti-C5 mAb, along with 8 age and sex-matched WT mice treated with isotype control, D1.3. Kaplan Meier survival curve; P = 0.8270 (Log-rank (Mantel-cox) test). Median survival WT = 29.5 days, WT + anti C5-mAb = 35.5 days.

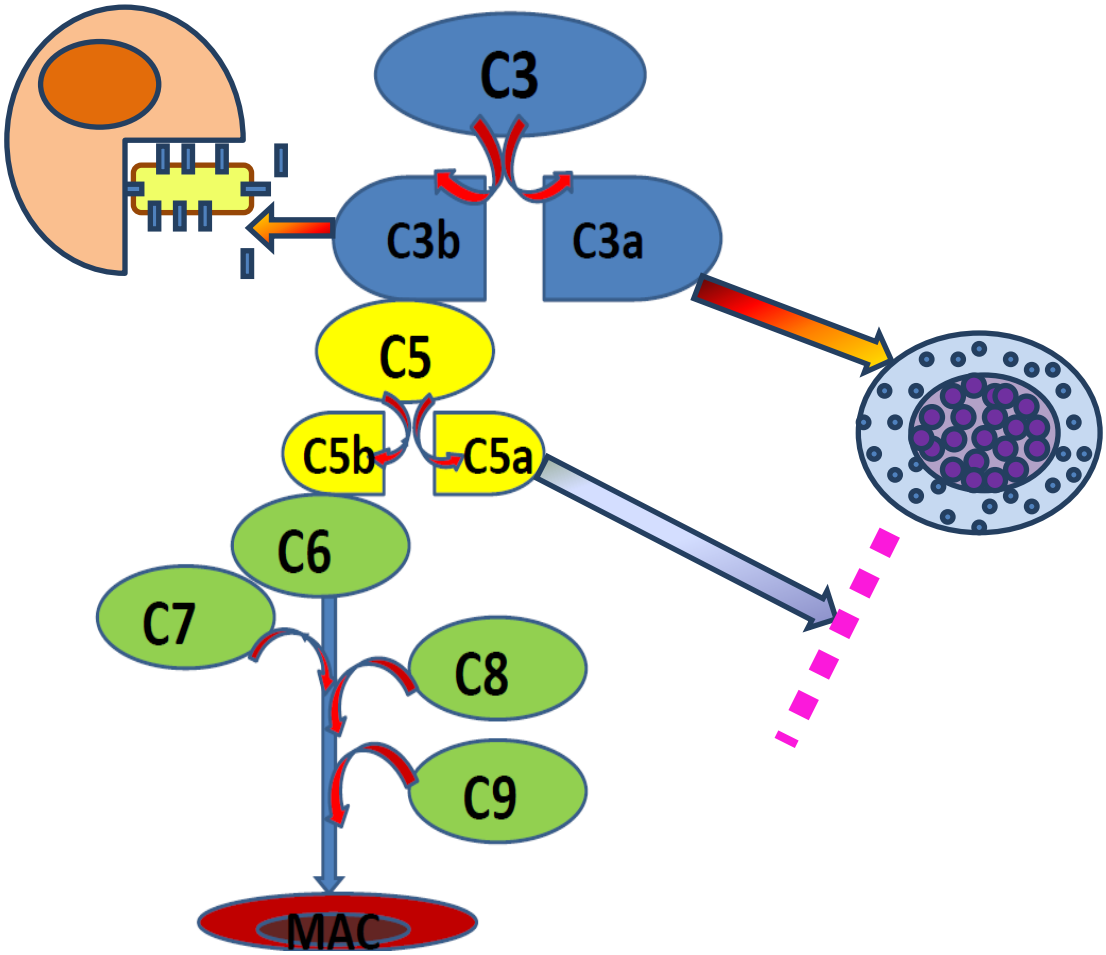


Figure 4.22: Phenotype achieved by the C5a receptor antagonist PMX-53. Although mice treated with the C5a receptor antagonist PMX-53 could still produce C5a they lack the ability to respond to it via its main receptor C5aR. They are still able to form MAC and hence maintain their capacity for complement-mediated lysis. They also retain the ability to produce both the anaphylatoxin C3a and the opsonising protein C3b.

The C5a receptor antagonist (C5aRA) was used at the dose described in other studies (1mg per kg) in both a 3 times per week and a daily dosing regimen. Treatment commenced the day prior to administration of C1498 cells and continued for the duration of the experiment. Mice were imaged twice weekly from day 7.

As no difference was observed between thrice weekly vs daily dosing, the results of the two experiments were combined. Results demonstrating no difference in survival C5aRA treated and control mice P = 0.3958, median survival WT = 25.5 days, WT + C5aRA = 24 days (Figure 4.23). It was however noted that approximately 20% C5aRA treated mice failed to develop detectable disease.

C3a anaphylatoxin

Though several studies have explored the role of 'complement anaphylatoxins' in tumour progression, this has either been investigated via complete complement inhibition (C3-/- mice) or by therapeutic or genetic ablation of the C5aR (reviewed in(Sayegh, Bloch et al. 2014)), with little attention being paid to the specific role of C3a.

It was therefore hypothesised that whilst C5a has a role in cancer progression in other tumour models, C3a might be more important in the case of AML. The effect of C3a-C3aR signalling on the progression of AML was therefore explored using genetically modified mice that did not express the C3a receptor (C3aR-/-). These mice are able to produce but not respond to C3a due to the lack of C3aR expression (Figure 4.24).

These studies revealed that AML in a C3aR-/- mouse had a more aggressive phenotype than observed in control mice, P<0.0064**, median survival WT = 25.5 days, C3aR-/- = 20 days (Figure 4.25). This was initially a somewhat unexpected finding as it suggests that C3a-C3aR signalling protects against progression of AML,

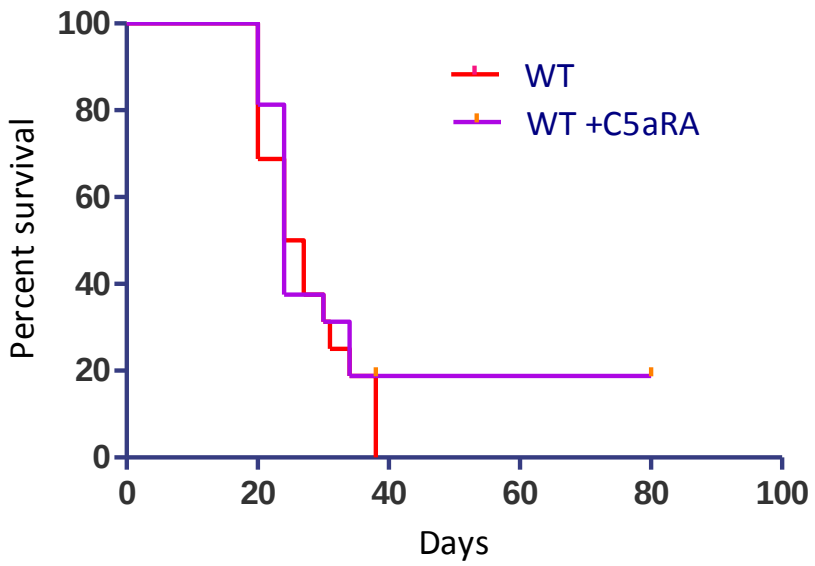


Figure 4.23: Survival of WT mice treated with C5a receptor antagonist. 5 x 10^6 C1498 cells injected IV into a total of 16 WT mice treated with the C5a receptor antagonist (C5aRA) PMX-53 along with 16 age and sex-matched WT controls. Kaplan Meier survival curve; P = 0.3958 (Log-rank (Mantel-cox) test). Median survival WT = 25.5 days, WT + C5aRA = 24 days. It was however noted that approximately 20% C5aRA treated mice failed to develop detectable disease.

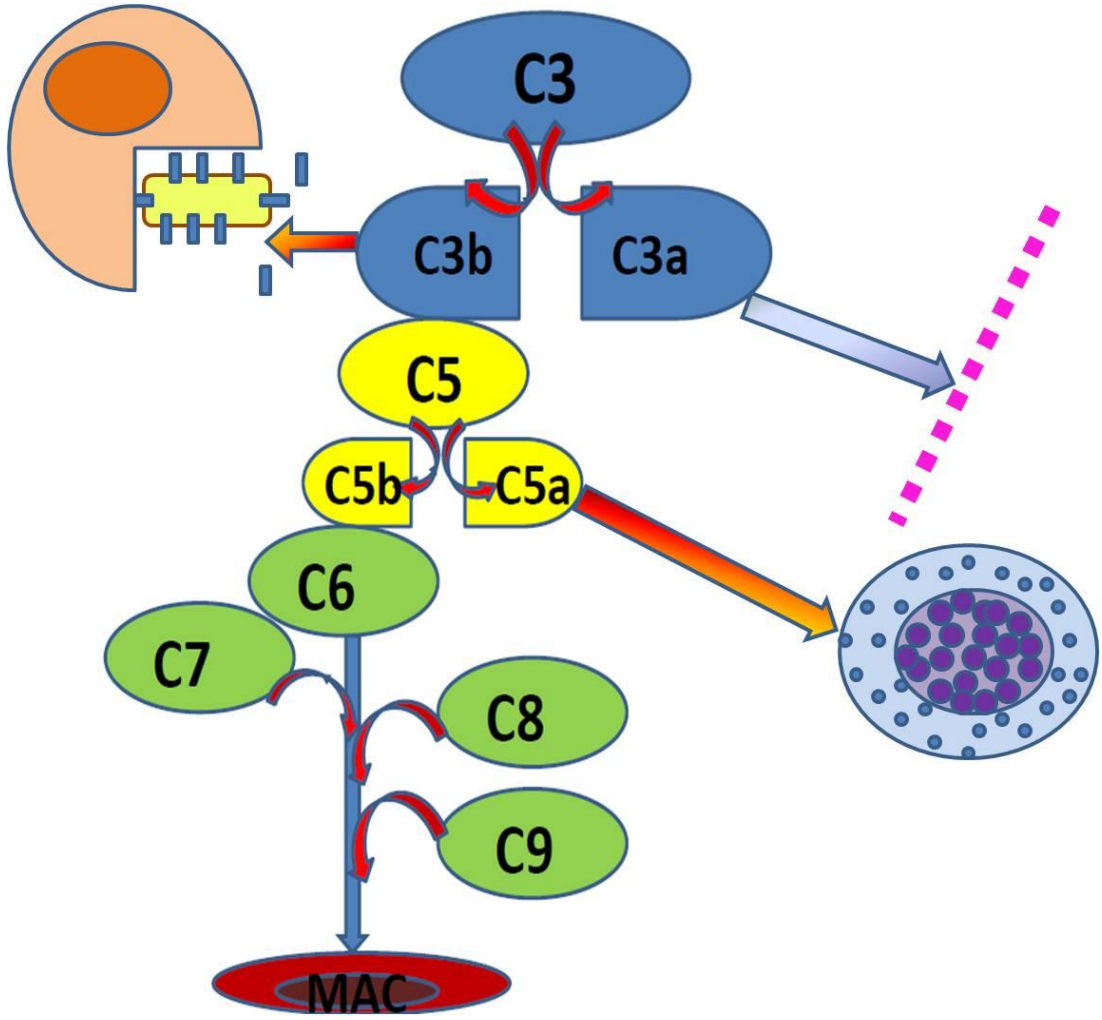


Figure 4.24: Phenotype of C3aR deficient (C3aR-/-) mice. C3aR-/- mice are still able to produce C3a but are unable to respond via the C3a receptor. They can still form MAC and hence maintain their capacity for complement-mediated lysis. They also retain the ability to produce both the anaphylatoxin C5a and the opsonising protein C3b.

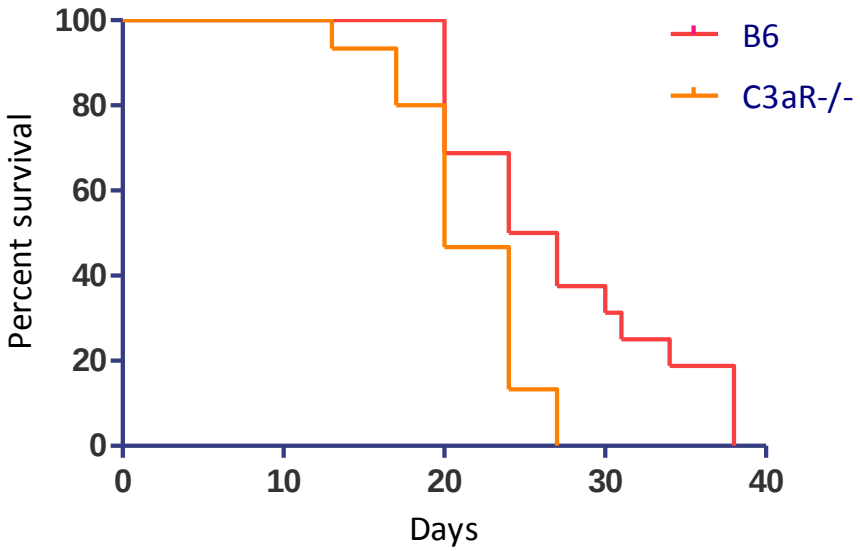


Figure 4.25: Survival of C3aR-/- vs WT mice. 5 x 10^6 C1498 cells injected IV into a total of 16 WT and C3aR-/- mice. Kaplan Meier survival curve; P < 0.0064** (Log-rank (Mantel-cox) test). Median survival WT = 25.5 days, C3aR-/- = 20 days.

and therefore could not account for the protective phenotype observed in C3-/- mice.

A review of the literature revealed that C3a has been shown to have an opposing role to C5a in both sepsis and an acute inflammatory response (Hollmann, Mueller-Ortiz et al. 2008; Wu, Brennan et al. 2013). During the acute inflammatory response C3a has a potent anti-inflammatory role acting in direct opposition to C5a via inhibiting the mobilization of neutrophils into inflamed tissues (Wu, Brennan et al. 2013). In sepsis, whilst C5aR signalling is deleterious, C3aR signalling is protective (Hollmann, Mueller-Ortiz et al. 2008). These findings suggest that C3a might regulate C5a generated inflammatory responses. Signalling via the C3a receptor could thus have a protective role in AML contrary to the deleterious role for C5a described in other malignancies. An alternative explanation would be that C3a produced by the host is signalling via an alternative receptor such as C5aR2 or driving proliferation of malignant cells via interactions with their C3aR (discussed in depth later).

As no protection against the progression of AML was observed via impinging on either the C5a-C5aR or C3a-C3aR signalling pathways, I postulated that the pathways compensate for each other thus should be blocked concurrently. To achieve this, C3aR-/- mice were treated with the C5aR antagonist described above. Treatment with C5aRA had no impact on the phenotype of C3aR-/- mice (Figure 4.26). P = 0.2378, median survival: C3aR-/- = 24 days, C3aR-/- + C5aRA = 25.5 days. This is in line with the absence of effect observed when C5aRA was administered to WT mice. However, as this was only investigated in one experiment (8 vs 8) this finding needs to be confirmed.

Whilst the use of genetically modified C3aR-/- mice impinges C3a-C3aR signalling via host cells, it has no effect on C3a production. In addition, the absence of the C3aR on host tissue might result in higher plasma levels of C3a in C3aR-/- mice. Hence C3a produced by the host mice could still have significant biological effects. The C3a cleavage product C3a desArg binds to and mediates its effect via the

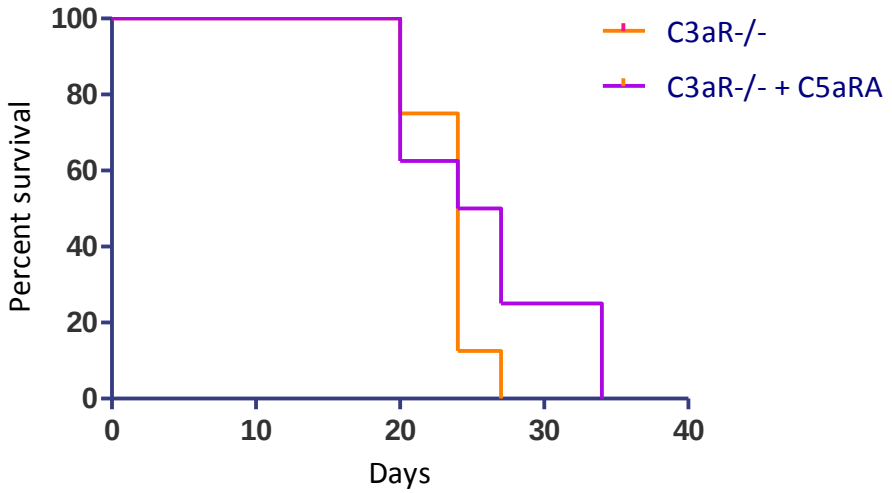


Figure 4.26: Survival of C3aR-/- mice +/- treatment with C5aRA. 5 x 10^6 C1498 cells injected IV into a total of 8 C3aR-/- mice treated with the C5aRA compared to 8 age and sex-matched C3aR-/- controls. Kaplan Meier survival curve; P = 0.2378 (Log-rank (Mantel-cox) test). Median survival C3aR-/- = 24 days, C3aR-/- + C5aRA = 25.5 days.

alternative C5aR C5aR2 (Kalant, Cain et al. 2003; Li, Coulthard et al. 2013). However, as C3aR-/- mice do not express C5aR2 (unpublished data, private correspondence with Prof Craig Gerard) this rules out an effect of downstream C3a cleavage products on this receptor. However it is possible that C3a might signal via either C3aR or C5aR2 expressed on the administered AML cells and hence impact on their survival and proliferation.

In contrast to the use of a genetically modified animal, treatment of WT mice with C5aRA will impinge on both host and malignant cell receptors. It will however have no effect on C5a signalling via the alternative C5a receptors C5aR2. Hence, any effects mediated through C5a signalling via C5aR2 would be unaffected (discussed in detail later in this chapter).

4.2.5.2 Complement-induced protection could be an *in vivo* phenomenon

A longstanding assumption that complement facilitates the elimination of cancer cells (Rutkowski, Sughrue et al. 2010) has been reinforced by early promising results in studies using monoclonal antibodies against cell-associated complement inhibitors in order to enhance anti-tumour complement dependent cytotoxicity (Gelderman, Kuppen et al. 2002; Sier, Gelderman et al. 2004; Allendorf, Yan et al. 2005). This longstanding dogma suggesting that activation of the complement system is protective against progression of cancer has been challenged by recent publications demonstrating that complement actually impinges on the immune response to cancer via the release of the anaphylatoxin C5a (Markiewski, DeAngelis et al. 2008). Despite a profound survival advantage being observed in C3-/- mice, no effect of C5a or C5aR manipulation has been observed in the C1498 AML model. Mice genetically deficient for C3aR also failed to provide an explanation for the protective phenotype observed in C3-/- mice.

Whether the production of MAC itself had any effect on progression of AML was next explored. If the longstanding dogma of MAC being able to clear malignant cells and thus protecting against development/progression of tumours is true, then a

more aggressive phenotype would be observed. However, it is also possible that complement-induced protection could play a role *in vivo.* Sub-lytic doses of MAC could thus protect malignant cells *in vivo* against lytic doses of not only complement but also other pore-formers including perforin the cytolytic mechanism utilised by both NK and CD8+ T cells. If this is the case, then the presence of MAC would promote tumour growth and conversely an inability to produce MAC would result in protection from progression of AML.

C6-/- mice

C6 knock-out (C6-/-) mice, which had been back crossed onto a B6 background ten times, were used to assess the role of MAC in development of AML. The C6-/-mouse is unable to produce C6 and hence MAC, and therefore lacks any capacity for complement-mediated lysis. However, they have a fully functioning C1 – C5 complement system and therefore producing both C5a and C3a anaphylatoxins and the opsonising protein C3b (Figure 4.27). If MAC is effective at eliminating tumour cells *in vivo*, a more rapid progression of leukaemia would be expected. If complement-induced protection plays a role *in vivo,* then a lower incidence of leukaemia would be observed.

The development on AML was assessed in a total of 38 C6-/- mice, 14 male and 24 female, over the course of 4 separate experiments. Compared to age and sex-matched controls, C6-/- mice exhibited a significant survival advantage, P= <0.0001. Median survival: WT = 27 days, C6-/- = 35.5 days. Approximately 25% of C6-/- mice remained free from AML (Figure 4.28). It should be noted that though the absence of MAC appears to be protective, this does not fully account for the protective phenotype observed in C3-/- mice as 75% of C6-/- mice did develop AML (compared to just 9% of C3-/- mice).

It is somewhat surprising that the protective phenotype observed in C6-/- mice was not observed in WT mice treated with anti-C5 mAb, which should also be unable to form MAC. This could be the result of an incomplete blockade of MAC at the tissue

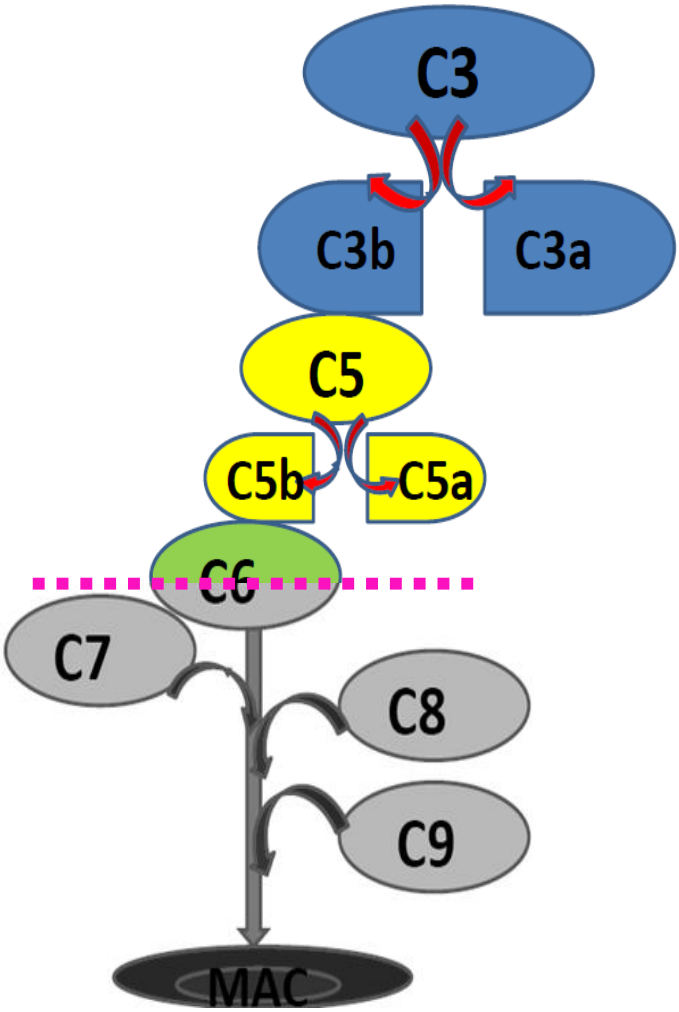


Figure 4.27: Phenotype of C6 deficient (C6-/-) mice. C6-/- mice are unable to form MAC and therefore lack any capacity for complement-mediated lysis. They retain the ability to produce both C3a and C5a anaphylatoxins and the opsonising protein C3b.

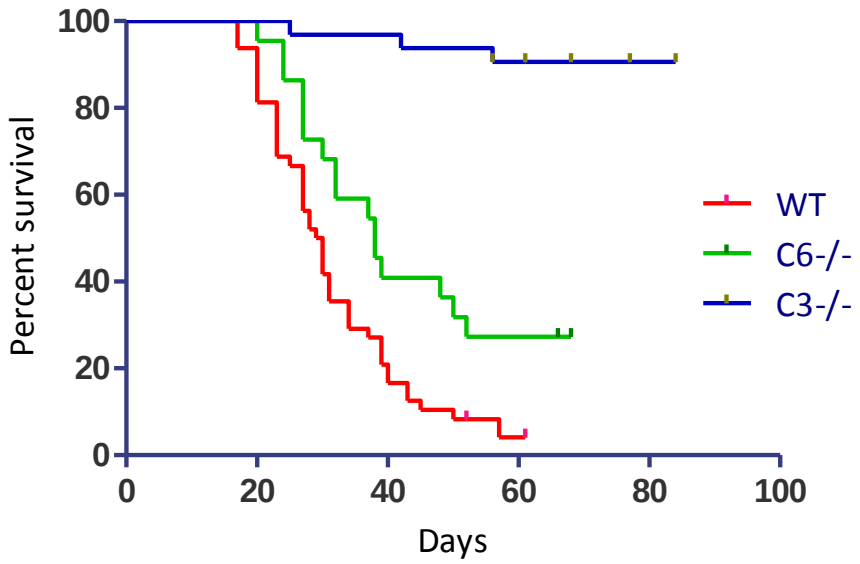


Figure 4.28: Survival of C6-/- vs WT and C3-/- mice. 5 x 10^6 C1498 cells injected IV into a total of 48 WT and 38 C6-/- mice. Kaplan Meier survival curve; P < 0.0001*** (Log-rank (Mantel-cox) test). Median survival WT = 27 days, C6-/- = 35.5 days. Approximately 25% of C6-/- mice failed to develop detectable disease.

level due to either incomplete tissue penetration of the antibody or to constant local production of complement proteins in the tumour microenvironment. This requires further investigation. Though not affecting survival, the use of the anti-C5 mAb did appear to lead to an altered disease distribution in line with C6-/- mice which will be discussed in detail later.

C6 antisense oligonucleotide

In order to confirm the finding in C6-/- mice, an antisense oligonucleotide which targets C6 mRNA in order to prevent the synthesis of C6 in the liver and hence the production of MAC was used to reduce C6 synthesis in WT mice. The C6 antisense along with a control oligonucleotide was kindly provided by Professor Kees Fluiter (Fluiter, Opperhuizen et al. 2014). A dose of 5mg/kg subcutaneously (SC) three times a week was used, in line with his recommendation. This was commenced the day prior to administration of 5×10^6 C1498 cells IV.

No survival advantage was observed in mice treated with the C6 antisense vs the control oligonucleotide. P = 0.6688, median survival: WT + control oligonucleotide = 28.5 days, WT + C6 antisense oligonucleotide = 24 days (Figure 4.29).

Plasma samples harvested from mice treated with either the C6 antisense or control oligonucleotide were sent to Professor Fluiters laboratory for measurement of C6 levels. This showed that C6 levels were successfully suppressed to a level of between 0.3-1.5µg/ml (mean 0.7µg/ml) in treated mice compared to 4.5- 9.4µg/ml (mean 6.5µg/ml) in control animals (Figure 4.30). Though the inhibition of C6 production was successful, it is not complete and might mean that a sub-lytic level of MAC could still be achieved in C6 low mice. It is also possible that reducing C6 in this way results in tumour cells being even more prone to sub-lytic MAC.

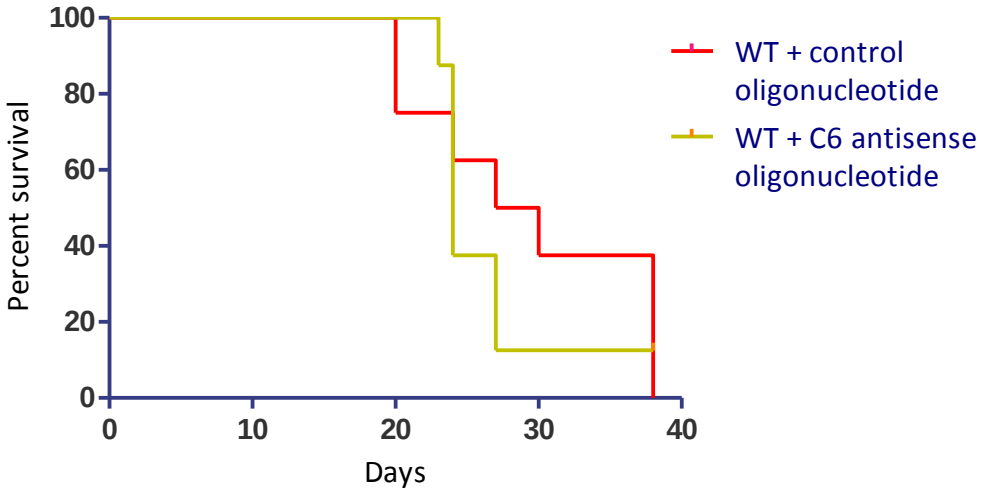


Figure 4.29: Survival of WT mice treated with C6 antisense oligonucleotide. 5 x 10^6 C1498 cells injected IV into a total of 8 WT mice treated with a C6 antisense oligonucleotide along with 8 age and sex-matched WT controls. Kaplan Meier survival curve; P = 0.6688 (Log-rank (Mantel-cox) test). Median survival WT = 28.5 days, WT + C6 antisense oligonucleotide = 24 days.

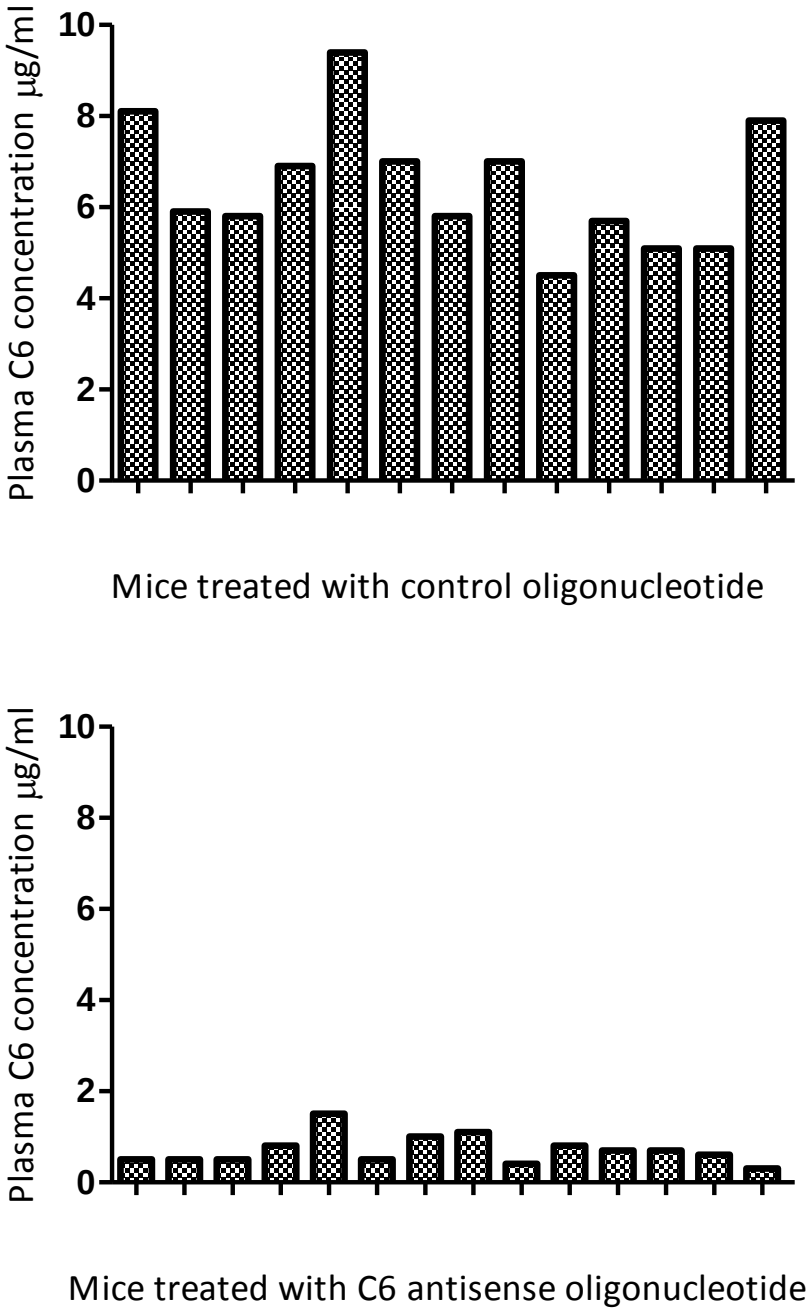


Figure 4.30: Plasma C6 levels (µg/ml). Plasma harvested from mice at the end of the protocol was sent to Professor Fluiters laboratory for measurement of C6 plasma levels. C6 levels from WT mice treated with either control (top) or C6 antisense (bottom) oligonucleotide are shown.

C5a receptor antagonism in C6-/- mice

Approximately 20% of C5aRA treated mice remained tumour-free. Although not statistically significant, this is similar to 25% tumour-free C6-/- mice. It is also possible that progression of AML in a WT mouse might be too aggressive for any protective effect conferred by treatment with C5aRA to be detected. C5aRA might therefore be more effective in the setting of less aggressive disease. An additive or even synergistic effect might also be observed in the absence of MAC. The effect of C5aRA on disease progression in C6-/- mice was therefore explored.

In two separate experiments with a total of 16 mice, no differential survival was observed between C6-/- mice treated or not with the C5aRA using either thrice weekly or daily dosing schedules. P= 0.4958, median survival: C6-/- = 34 days, C6-/- + C5aRA = 34 days (figure 4.31). This supports previous findings in both WT and C3aR-/- mice, which demonstrated that C5a receptor antagonism also had no impact on disease progression in this model.

4.2.5.3 Tumour distribution

Aside from survival, illustrated using Kaplan Meier survival curves and statistical significance, assessed by log-rank (Mantel-Cox) tests, manipulation of the complement system also appeared to alter disease distribution. This was explored by comparing tumour distribution from 3 experiments which included WT and C6-/- mice +/- treatment with C5aRA or anti-C5 mAb.

The classical tumour distribution observed when C1498 AML is administered IV to WT mice is the development of central tumours affecting sites such as the liver, spleen and ovaries with only occasional mice developing peripheral lesions on their back or limbs- most of these in conjunction with central tumours. It was noted that in contrast to this, mice that are unable to produce MAC, either due to C6 deficiency or through neutralisation of C5 by anti-C5 mAb, developed less central

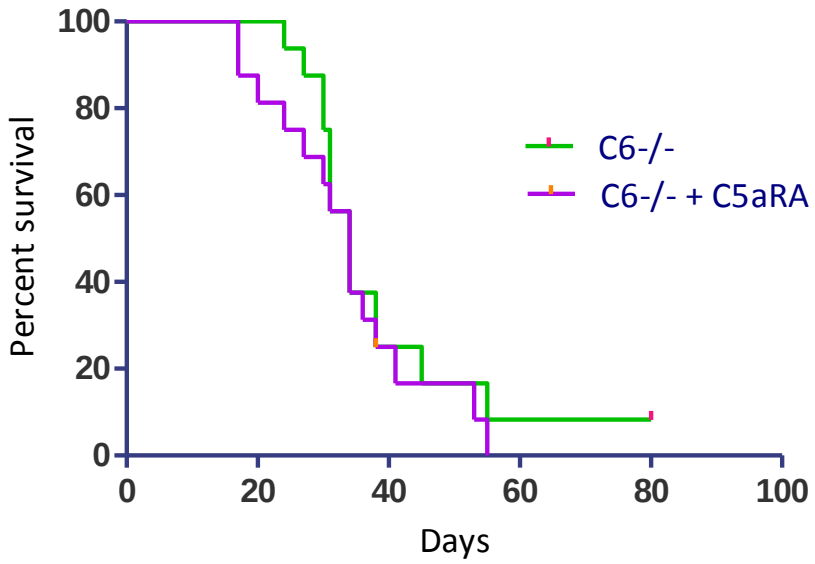


Figure 4.31: Survival of C6-/- mice treated with the C5a receptor antagonist. 5 x 10^6 C1498 cells injected IV into a total of 16 C6-/- mice treated with C5aRA along with 16 age and sex-matched C6-/- controls. Kaplan Meier survival curve; P = 0.4958 (Log-rank (Mantel-cox) test). Median survival C6-/- = 34 days, C6-/- + C5aRA = 34 days.

but more peripheral tumours. In the case of C6-/ mice, approximately 40% failed to develop central tumours whilst most of these went on to develop peripheral tumours, in some C6-/- mice AML failed to progress at any sites. In anti-C5 mAb treated mice, whilst only around 1/3 developed central tumours, all remaining mice went on to develop peripheral tumours on their back or limbs. It was therefore concluded that the absence of MAC, conferred protection from the development of central but not peripheral tumours.

In contrast to this, it was noted that although approximately 80% of WT mice treated with C5aRA developed central tumours, none of the mice developed peripheral tumours, leading to 20% surviving long-term. Though isolated peripheral tumours are a rarity in WT mice, they are seen in combination with central tumours in approximately 20% of cases, making the observation observed in C5aRA treated mice potentially significant, however more studies are necessary in order to confirm this finding. Unfortunately, the potential protection conferred centrally, by the absence of MAC, and peripherally, by treatment with the C5aRA, do not appear to be cumulative with C5aRA treated C6-/- mice displaying an intermediate rather than additive phenotype (Figure 4.32).

4.2.5.4 Cell adherence and migration

When tumour cells enter the bloodstream they bind to and activate platelets and leukocytes which facilitate tumour cell arrest at the vessel wall and extravasation into tissues (reviewed in (Gay and Felding-Habermann 2011)). C3 activation can enable tethering between activated platelets and polymorphonuclear leukocytes via iC3b and the complement receptor CR3 (CD11b/CD18, Mac-1) (Hamad, Mitroulis et al. 2015). The murine AML cell line C1498 has been shown to express several adhesion molecules including CD11b (Barrett and Jiang 2000). CD11b expression also predicts poor prognosis in human AML (reviewed in (Xu, Li et al. 2015)). It was therefore possible that complement, specifically iC3b, may play a role in the ability of C1498 cells to tether and migrate. If this was the case then C1498 might be

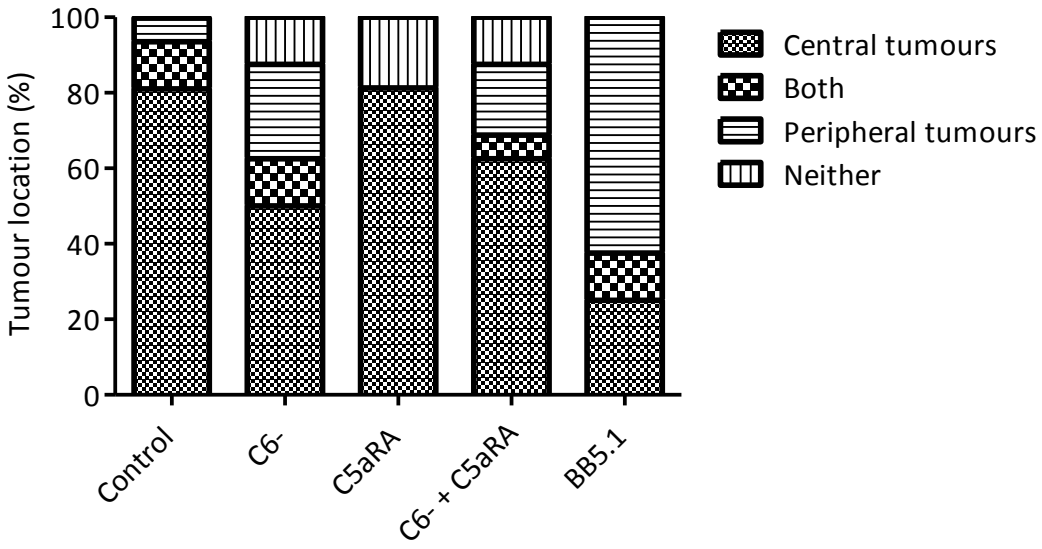


Figure 4.32: Tumour distribution in mice lacking various complement components. It was noted that along with effects on survival, manipulation of complement also altered disease distribution. When mice were unable to produce MAC either due to a genetic deficiency or use of anti-C5 mAb, they developed less central but more peripheral tumours. In contrast, mice receiving C5aRA developed a similar proportion of central tumours but no peripheral tumours. Administering C5aRA to C6-/- mice resulted in an intermediate (rather than additive) phenotype. The phenotype displayed by C5aRA treated C6-/- mice was also not analogous to WT mice treated with anti-C5 mAb.

unable to enter tissues in complement deficient mice, remaining within the vasculature and giving the immune system a better opportunity to clear tumour cells. Thus, the role of the immune system demonstrated thus far though still important, may be secondary to the influence of complement on tumour take.

In order to remove the need for the tumour cells to leave the blood stream and establish themselves within tissues, C1498 AML cells were administered to WT and C3-/- mice via subcutaneous (SC) injection. Tumour growth was then measured via calliper measurement alongside *in vivo* imaging.

Results of this study revealed that when the need for tumours to seed was removed, C1498 cells were able to grow at a similar rate in C3-/- and WT controls. However, tumour volume peaked at around day 7 before tumours in both C3-/- and WT mice began to regress. This timescale is consistent with the development of an effective T-cell response with an adaptive immune response generally being mounted after 4-7 days (Mandell, Bennett et al. 2000). Interestingly, whilst tumours then re-grew in the majority of WT mice, C3-/- mice cleared their tumours and remained tumour-free (Figure 4.33).

4.3 Discussion

4.3.1 T Cells are required to control growth of AML in C3-/- mice

The most significant finding is that AML fails to progress in a complement deficient environment (Figure 4.7). T cell depletion studies suggest that C3-/- mice are able to clear AML through a CD8+ T cell mediated mechanism (Figure 4.14). However, C3-/- mice do not appear to mount a superior memory T cell response as measured by IFN-γ response to exposure to irradiated tumour cells following vaccination (Figure 4.16). In fact, in line with other vaccination studies, C3-/- mice mounted a slightly inferior response to tumour cell vaccination than WT controls (Pishko, Kirimanjeswara et al. 2004; Fernandez Gonzalez, Jayasekera et al. 2008).

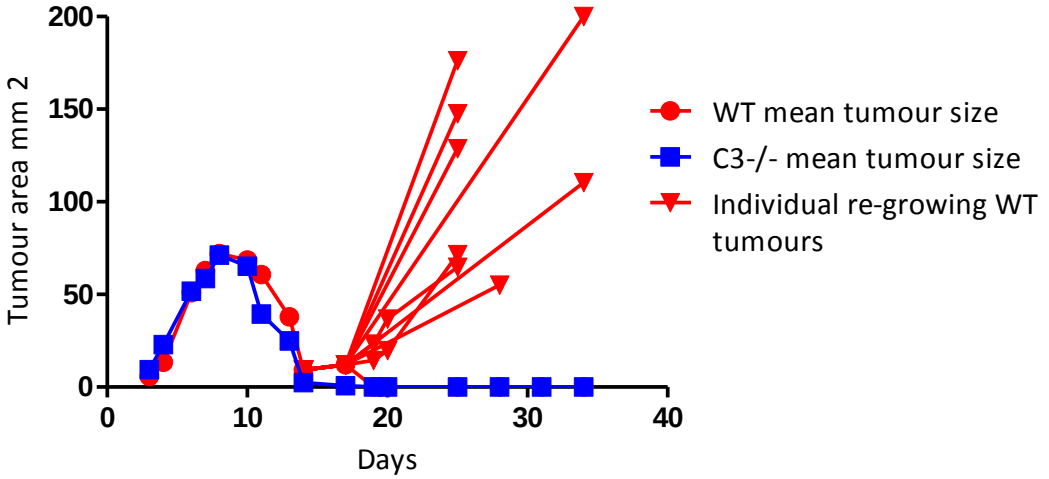


Figure 4.33: Growth of subcutaneous (SC) C1498 tumours in C3-/- vs WT mice. 10^6 C1498 cells were injected SC and tumour growth monitored. In contrast to IV inoculation both WT and C3-/- mice developed tumours at the injection site with initially comparable tumour growth. At around day 10 tumours began to recede in all mice, consistent with development of an anti-tumour T-cell response. Interestingly, whilst tumours re-grew in the majority of WT mice, all C3-/- mice cleared their tumours and remained tumour-free. WT n= 15, C3-/- n = 12.

4.3.1.1 Dose of C1498

Previous studies in WT mice have demonstrated that both NK and CD8+ CTLs are able to lyse C1498 however, only NK cells were shown to be effective *in vivo* (Boyer, Orchard et al. 1995). Data from other groups demonstrate a trend towards enhanced AML progression following combined depletion of CD8+ and CD4+ T cells, however this did not reach statistical significance (P= 0.1, n = 16). A very low dose of C1498 (10^4) was used in these studies meaning that the threshold level of antigen required in order to trigger an adaptive immune response may not have been reached with the innate immune system effectively clearing low doses of tumour cells without triggering an adaptive immune response(Boyer, Orchard et al. 1995). Experiments described in this thesis show that CD8+ T cell depleted WT mice receiving 5 x 10^6 C1498 cells IV had a significantly worse survival than WT controls (P<0.0001, n= 8, Figure 4.13). This suggests that a large exposure to C1498 is capable of inducing a CTL response. This response is however ineffective at clearing AML in a complement sufficient environment. In contrast the CTL response in complement deficient mice is capable of clearing AML and preventing disease progression.

4.3.1.2 Route of administration

It has previously been demonstrated that T cell responses are different when AML cells are delivered via a SC vs IV route. SC or IP but not IV immunization with irradiated C1498 cells resulted in immune resistance to subsequent live C1498 innoculation (Boyer, Orchard et al. 1995). In addition, IV inoculation has been shown to result in the development of immune tolerance, preventing functional T cell activation following a subsequent SC AML cell challenge (Zhang, Chen et al. 2013). This T cell tolerance was antigen specific and independent of Tregs and MDSCs (Zhang, Chen et al. 2013). Despite the lack of immunogenic response observed when C1498 is administered IV, the high dose of C1498 cells used in my studies appears to have been sufficient to trigger a CD8 mediated immune response demonstrated by the significantly worse survival seen in CD8-depleted vs control

WT mice. Contrary to the inefficient immune responses demonstrated in WT animals, who almost universally succumbed to the progression of AML, in complement deficient animals the immune response was sufficient, even in the face of a large and highly aggressive tumour load, to prevent the progression of AML.

Injection of the tumour cells SC also appears to induce an immune response against C1498 tumours in both C3-/- and WT mice. Yet, whilst the tumours in the majority of WT mice escaped from this immune control, C3-/- mice successfully cleared tumours and remained disease free (Figure 4.32). This demonstrates the primary CD8 T cell response to be a critical time-point at which complement impacts on immune control of AML. It also implies that complement is involved in immune escape.

ELISpot analysis of IFN-γ responses generated following injection of mice with irradiated tumour cells demonstrated that memory T cell responses in C3-/- mice were not superior to those observed in WT mice (Figure 4.16). In fact C3-/- mice appeared to mount a slightly inferior response to tumour vaccination than WT controls, a finding supported by other studies of the efficacy of vaccination in C3-/- mice (Pishko, Kirimanjeswara et al. 2004; Fernandez Gonzalez, Jayasekera et al. 2008). As the number of splenocytes was standardised in these assays it is unknown whether C3-/- mice had a higher number or proportion of CD8+ T cells following vaccination than WT controls. It is therefore conceivable that C3-/- mice produce higher numbers rather than more effective CD8+ T cells which could account for the more effective CD8+ T cell mediated immune response.

4.3.1.3 IFN-γ independent CD8+ T cell killing

Whilst ELISpot assays have thus far have failed to demonstrate any effect, this assay did not investigate other readouts of T cell activity such as killing or production of cytokines such as IL-2 or TNF-α. Therefore it is possible that complement suppresses these functions of CD8+ T cells which would not be revealed in an IFN-γ- based ELISpot. Fluorospot assays which differ from ELISpots in that they use multiple

fluorescent labelled anti-cytokine detection antibodies to allow optimal measurements of multiple cytokines or granzymes, could be used. As well as allowing fuller characterisation of T cell responses in C3-/- vs WT mice, these studies could also be extended to investigate T cell responses in other genetically altered mouse models such as C6-/- and C3aR-/- mice.

4.3.1.4 Immune response to irradiated vs live cells

Observed effects could also be a result of differences between immune responses to vaccination with irradiated tumour cells vs inoculation with live cells. Apoptotic tumour vaccination, induced by using γ-irradiation prior to injection, has been shown to result in potent immune responses *in vivo* with strong CD4+ and CD8+ T cell responses observed at tumour sites (Scheffer, Nave et al. 2003). In my studies, following vaccination with irradiated tumour cells, both WT and C3-/- mice mounted a tumour specific IFN-γ response. Somewhat surprisingly, given the results of my T cell depletion studies, the immune response to vaccination was no superior in C3-/- vs WT mice.

Prophylactic tumour vaccination against subsequent live tumour challenge depends on the ability of professional APCs such as dendritic cells (DCs) to process tumour antigens from whole cells and present them to CD4+ and CD8+ T cells. Exposure to live cells has been demonstrated to be significantly better at inducing immune responses than immunization with irradiated cells (Matheoud, Perie et al. 2010; Volovitz, Marmor et al. 2011). Cross-presentation by DCs of antigens from live cells was demonstrated to induce stronger natural CD8+ T-cell responses against lethal tumour challenge than those induced by irradiated tumour cells resulting in stronger IFN-γ responses and complete protection against tumours (Matheoud, Perie et al. 2010). Hence the better immune response observed in C3-/- mice following exposure to live inoculums of leukaemia could be due to critical differences in immune responses following exposure to apoptotic (irradiated) vs live cells.

4.3.1.5 The effect of complement on primary CD8+ T cell response

It is also possible that complement may impinge on T cell responses to live cell inoculation whilst not affecting memory responses induced by vaccination with irradiated cells. This could be seen as analogous, but opposite, to the effect of CD4+ T cells on CD8+ memory T cell responses. CD4+ T cells, whilst not critical to successful primary CD8+ T cell responses, are essential in order to generate memory CD8+ T cell response (Bourgeois and Tanchot 2003; Janssen, Lemmens et al. 2003; Shedlock and Shen 2003). Conversely, complement might be critical to the primary T cell response but fail to play a role in the generation of a memory response.

Further experiments are required to address whether complement impinges broadly on CD8+ T cell responses or whether the effect is limited to the primary response. Previous studies using *in vitro* generated CTLs have failed to control established AML *in vivo (Zhou, Bucher et al. 2009).* An investigation of the role of complement on tumour-specific CD8+ T-cells might offer an explanation for the disparities observed between *in vitro* vs *in vivo* studies. These studies would determine whether AML-specific CTLs stimulated in a complement sufficient environment are inferior to those stimulated in a complement deficient environment by generating AML-specific CTL lines from C3-/- and WT mice for reinfusion into complement–deficient and sufficient CD8-depleted mice injected IV with AML cells. These experiments would confirm whether memory T cell responses against AML were any different when raised in a complement deficient vs sufficient environment *in vivo.* It could also offer insight into whether complement could have a specific effect on primary T cell responses. If CTL therapy is both ineffective and is impinging on a more robust primary response in C3-/- mice, then increased progression of AML might be observed in C3-/- mice following vaccination.

T cell-tolerance leading to inefficient clearance of AML cells has been shown to be dependent on a lack of CD40 mediated activation of host antigen presenting cells (APCs) (Zhang, Chen et al. 2013). Administration of agonistic anti-CD40 Ab, to leukaemia-bearing mice, activated host APCs, enhanced accumulation of functional

T cells and prolonged survival (Zhang, Chen et al. 2013). However, this is unlikely to be a mechanism through which complement impinges on primary T cells responses to leukaemia as both C3a and C5a have been demonstrated to enhance the function of dendritic cells (DCs) during T cell priming (Sacks and Zhou 2012).

Tregs alter their phenotype depending on the concomitant inflammatory response (Yamaguchi, Wing et al. 2011)- an environment which will be substantially altered in the absence of complement. A role for complement in negatively regulating immune response has however been demonstrated via induction of Treg responses. Stimulation of the C3 regulator MCP (CD46) steers human CD4+ T cells towards an immunosuppressive role characterised by the production of interleukin-10 (IL-10)(Kemper, Chan et al. 2003). This finding is supported by the low numbers of Tregs observed in human C3 deficiency (Ghannam, Pernollet et al. 2008). Despite a recognised key role for complement in promoting graft rejection responses to both major and minor histocompatibility antigens, a contradictory role exists whereby complement inhibits rejection and thus promotes graft tolerance in the setting of minor histocompatibilty antigen mismatch (Sacks and Zhou 2012). Increased rejection in C3-/- female mice receiving skin grafts from male donors is coupled with an increase in antigen specific CD8+ IFNγ T cells (Baruah, Simpson et al. 2010). In a mouse model of graft tolerance induction, transplant tolerance was shown to be dependent on C3, with FOXP3 + Tregs failing to suppress the immune response in the absence of complement (Sacks and Zhou 2012). It might therefore be most likely that complement is impinging on adequate immune responses to AML via the induction and/or activation of Tregs.

4.3.1.6 Immunosuppressive mechanisms induced by AML

Failed vaccination attempts in mice with established leukaemia have led to the identification of several mechanisms by which C1498 leukaemia suppresses immune responses including increasing Treg and MDSC activity. Interestingly, although the proportion of MDSCs is increased in spleens from leukaemic animals where they suppress the activity of CD4+ T cells, they do not appear to suppress CD8+ T cells

(Gibbins, Ancelet et al. 2014). Recruitment of MDSC may therefore be irrelevant in the AML model described herein explaining why neutralising C5 failed to prevent progression of the disease.

Progression of C1498 AML leads to an accumulation of regulatory T-cells (Treg) at disease sites (Zhou, Bucher et al. 2009). Treg depletion alone is ineffective at clearing tumours, requiring concurrent transfer of anti-AML CTLs (Zhou, Bucher et al. 2009). Based on the data described herein, it is tempting to speculate that combining Treg- and complement-depletion might prove an effective way of optimising both natural and induced CTL responses.

The SC model could be used in order to further explore immune escape, observed in WT but not C3-/- mice. Immunohistochemistry could first be used to investigate tumour infiltration by immune cells. This would identify significant differences in the immune cell infiltrates between receding vs re-growing tumours in WT mice and between receding tumours in WT vs C3-/- mice in order to identify which cell sub-types are key to successful immune mediated clearance and the avoidance of immune escape (tissue samples already stored). Fluorospot assays to measure AML-specific T-cell responses in mice could be used alongside immunophenotyping by multiparameter flow cytometry, in order to compare kinetics, quality and magnitude of T-cell responses in complement sufficient versus deficient mice. Adoptive transfer of cells identified to play a key role, between C3-/- and WT mice would confirm key mechanisms at play.

Whilst the experiments discussed above point to a role for the immune system in controlling AML, particularly in C3-/- mice, the experiments did not convincingly reveal that more efficient CD8+ T cell responses were generated in the absence of complement. Indeed, the entire dataset accumulated thus far is compatible with the hypothesis that whilst a CD8 T cell response is required for clearing the AML cells in C3-/- mice, another mechanism also contributes to the lack of tumour growth in these animals. This led us to investigate whether C3 has a role in seeding and tumour invasion.

4.3.2 The Role of complement in seeding of AML cells

Tumour cells need to bind and activate platelets and leukocytes in order to adhere to vessel walls and extravasate into tissues (Gay and Felding-Habermann 2011). C3 activation is required for tethering between activated platelets and polymorphonuclear leukocytes (Hamad, Mitroulis et al. 2015). It was therefore possible that complement activation has a key role in the ability of C1498 cells to tether and migrate. In a complement deficient environment AML cells might thus be unable to leave the circulation, create a favourable niche and proliferate leaving them in a vulnerable position. A prolonged presence within the circulation might provide circulating immune cells with an antigen load capable of triggering a more robust immune response. The absence of pro-survival signals provided by a tumour niches might also result in cell death independent of immune response. Hence, the role of the immune system may be secondary to the influence of complement on tumour take.

The main receptor for inactive C3b (iC3b) is complement receptor 3 (CR3) also known as CD11b/CD18 and Mac-1. It is binding via this receptor, following complement activation, which is essential for tethering between activated platelets and polymorphonuclear leukocytes (Hamad, Mitroulis et al. 2015). In the absence of C3, C1498 might be unable to tether to platelets, adhere to vessel walls and migrate into tissues. When injected IV, C1498 may thus remain circulating in the blood stream potentially giving the immune system a better chance of tumour clearance.

In order to remove the need for cells to leave the circulation and seed in tissues, C1498 cells were administered subcutaneously (SC). This resulted in comparable tumour growth in C3-/- and WT mice (Figure 4.32) suggesting that complement is required in order for C1498 AML cells to seed from blood vessels into tissue.

AML-niche interactions have a key role in development, progression, chemotherapy resistance and relapse in AML (Rashidi and DiPersio 2016). Myeloid blasts express a

range of adherence receptors that could be influenced by the presence of complement in the microenvironment. AML cells utilise the CXCR4/ CXCL12 axis in order to migrate, mobilise and home to disease sites (Kremer, Dudakovic et al. 2014). CXCL12 stimulation also results in enhanced adhesion of immature haematopoietic cells to endothelial cells and transendothelial migration(Peled, Kollet et al. 2000). E-selectin (ES) is involved in leukocyte rolling along luminal endothelial surfaces and the ES ligand, expressed on myeloid blasts, also mediates cell proliferation (Winkler, Barbier et al. 2012). The survival of myeloid blasts is also enhanced by their ability to bind to the vascular niche via ES ligands and activate pathways such as Wnt (Chien 2013). Blasts from relapsed or refractory AML have increased E-selectin expression indicating a possible role for vascular adhesion in resistant disease (Chien 2013). A role for complement in mediating the adherence and migration of leukaemia cells would thus be of critical importance.

It is possible that between IV inoculation with the cell line on day 0 and the induction of the immune response around day 10, C1498 cells might be readily detectable in the blood stream. Identification of cells circulating following IV injection, before they are cleared would support the seeding hypothesis. The seeding hypothesis could be further explored using the IV C1498 model via exploring the relative roles of complement, platelets and adherence molecules. Staining for platelets bound to the surface of circulating AML cells following IV administration to WT vs C3-/- mice would clarify whether platelets have a key role. Anti-platelet agents such as clopidogrel could be used to confirm the role of platelet aggregation on tumour cell take following IV administration. Small molecule inhibitors of adherence molecules such as E-selectin (GMI-1271) could also be utilised in my studies (Chien 2013).

4.3.3 C3 reconstitution/ depletion

I have demonstrated no evidence that C1498 cells produce C3 *in vitro* or *in vivo.* However, it remains important to address the possibility that C3 (whilst at undetectable levels) acts as an immunogen in C3-/- mice. An important next

experiment in order to confirm my findings would be to reconstitute the complement system in C3-/- mice using purified C3. C3 injected *in vivo* into C3-/- mice is only active for a limited time due to anti-C3 immune reactivity. Patterns of tumour growth following subcutaneous injection of cells clearly illustrates two key time-points at which C3 reconstitution in the IV model could be used to explore the relative importance of tumour cell seeding (day 0-7) and immune clearance (day 7-14).

Provisional attempts to reconstitute C3 in C3-/- mice using serum from WT mice administered daily to C3-/- recipients who had received C1498 cells IV, failed to result in any detectable disease. However significant limitations to this experiment included the availability of fresh serum and the permissible volume for injection. In future experiments, purified C3 should be prepared and used to determine whether reconstitution of C3 enables tumour growth in C3-/- mice.

Another method of determining the impact of complement on tumour growth is to deplete complement in WT animals. There are several methods of inhibiting complement *in vivo* though they all have limitations. The most widely used, cobra venom factor (CVF), is a structural and functional analogue of C3b which on contact with serum results in the formation of a long-lived C3/C5 convertase which activates C3 and C5 leading to soluble C5b-C9 formation and resulting in the depletion of serum complement activity (Vogel, Smith et al. 1984). CVF is short acting and highly antigenic, leading to a burst of C3a and C5a, making data interpretation challenging however it is widely used to assess the inhibitory effects of experimental anti-complement agents (Morgan and Harris 2003). The transient generalised complement inhibition achieved by this method has been demonstrated to impair tumour growth in a range of models (Janelle, Langlois et al. 2014; Downs-Canner, Magge et al. 2015).

4.3.4 Complement components key to the protective C3-/- phenotype

In order to determine the mechanism through which C3 deficiency promotes resistance to tumour growth the impact of various complement components was assessed (Figure 4.17). Complement deficient or therapeutically depleted mice were used in order to systematically investigate the role of complement in the progression of AML.

4.3.4.1 C5a

Results demonstrate no impact on survival in mice treated with either anti-C5 antibody or C5a receptor antagonist (Figures 4.21 and 4.23). Several studies have supported a role for complement in promoting tumour growth, via a range of mechanisms but often implicating C5a (Wang, Veeramani et al. 2009; Corrales, Ajona et al. 2012; Gunn, Ding et al. 2012; Nunez-Cruz, Gimotty et al. 2012; Janelle, Langlois et al. 2014; Vadrevu, Chintala et al. 2014; Downs-Canner, Magge et al. 2015; Imamura, Yamamoto-Ibusuki et al. 2015). Generation of C5a in the tumour microenvironment has been shown to promote the development of cancer by suppressing the anti-tumour CD8+ T cell-mediated response via the recruitment and augmentation of myeloid derived suppressor cells (MDSCs) (Markiewski, DeAngelis et al. 2008). Further work by the group has shown that the C5a receptor facilitates metastasis in breast cancer, though no effect on the primary tumour was observed. Metastasis was promoted via C5aR mediated suppression of both CD8+ and CD4+ T cell responses in the lungs via recruitment of MDSCs and regulation of their TGFβ and IL10 production (Vadrevu, Chintala et al. 2014). A separate group also demonstrated that C5a creates a favourable microenvironment for progression in lung cancer via both promoting angiogenesis and increasing MDSCs (Corrales, Ajona et al. 2012). Indeed, C5aR expression is more often seen on malignant cells at sites of metastasis than at the primary tumour site supporting a role in tumour progression (Imamura, Yamamoto-Ibusuki et al. 2015).

As discussed earlier, whilst MDSCs are increased in leukaemic animals and suppress the activity of CD4+ T cells, they did not appear to have a suppressive effect on CD8+ T cells (Gibbins, Ancelet et al. 2014). The protective effect of impinging on the C5aR in other disease models was mediated via suppressing MDSC activity against CD8+ T cells. If MDSCs present in leukaemic animals are truly unable to suppress CD8+ T cell activity, this mechanism might not be relevant in this setting.

There might be a more straightforward explanation for the absence of effect of impinging on C5a observed in my model. The same group which demonstrated the role of C5a in promoting tumour growth in an ovarian model, failed to demonstrate any effect on primary tumour growth in a breast cancer model, though an effect on priming the metastatic niche was observed (Markiewski, DeAngelis et al. 2008; Vadrevu, Chintala et al. 2014). They accounted this finding to a disparity in the rate of tumour growth between the models with harvested tumours in the breast cancer model being approximately 3 times bigger than observed in the TC1 ovarian model (Vadrevu, Chintala et al. 2014).

The absence of any impact on survival observed in my studies may therefore be due to the aggressive phenotype of the AML cells used. C1498 cells exhibit an exceptionally high ability to grow and migrate (Goldie, Butler et al. 1953). Tumour growth observed following the administration of C1498 leukaemia IV was rapid, diffuse and bulky and could therefore be beyond the capacity of any boost to CD8 function achieved by C5a inhibition. An exploration of the efficacy of the C5aRA in the setting of C1498 cells administered via a SC route, which might prompt a more efficient CD8+ T cell response, could be informative. The administration of lower doses of C1498 resulting in a smaller volume of tumour at the time of a CD8+ T cell mediated immune response might also reveal a role for C5a in the setting of a less insurmountable tumour load, though as discussed earlier, lowering the cell dose might result in the failure to trigger an adequate immune response.

The lack of an effect on tumour growth in mice treated with anti-C5 mAb may be due to failure to completely abrogate local production of C5a (even though

complete inhibition of complement mediated lysis was confirmed in the plasma of anti-C5 mAb treated mice (Figure 4.20)) or, in the case of the C5aRA, failure to block C5aR at the tumour site. Whilst other studies have used similar methods in order to demonstrate a role for C5a in tumour progression, the production of C5a at a local level, could have a more critical role in this disseminated leukaemic model (Lalli, Strainic et al. 2008; Strainic, Liu et al. 2008).

In addition to this, the role for C5a in tumour progression is still controversial. Whilst some studies indicate that C5a promotes progression through recruitment of MDSCs, C5a over-expression in tumour cells has been shown to result in tumour regression in a mouse model of breast cancer (Kim, Martin et al. 2005). Translational research also supports a positive role for C5a in anti-tumour monoclonal antibody therapy (Allendorf, Yan et al. 2005; Li, Allendorf et al. 2007; Fuenmayor, Perez-Vazquez et al. 2010). Hence the role for C5a in tumour progression remains controversial and my findings might not be surprising.

4.3.4.2 C3a

A role for C3a was also investigated. Mice deficient in the C3a receptor (C3aR-/-) were used in order to explore the role of C3a-C3aR signalling on progression of AML. Somewhat surprisingly, AML progressed more rapidly in mice lacking a C3a receptor (Figure 4.25).

This rapid progression of AML observed in C3aR-/- mice suggests a role for C3a signalling in limiting disease progression; a finding that seems to be at odds with the protective phenotype observed in C3-/- mice. Several explanations might account for this finding.

In contrast to C5a, in addition to its pro-inflammatory role, C3a also has an anti-inflammatory properties (Coulthard and Woodruff 2015). In the settings of both sepsis and an acute inflammatory response C3a has been shown to act in direct opposition to C5a (Hollmann, Mueller-Ortiz et al. 2008; Wu, Brennan et al. 2013). It

is therefore possible that C3a might have a protective role in the setting of malignancy. Recent studies have however found that contrary to this, C3a appears to have a deleterious role in malignancy (Guglietta, Chiavelli et al. 2016; Nabizadeh, Manthey et al. 2016).

Another possibility is that levels of C3a are elevated in C3aR-/- mice, due to absence of the receptor. As C3a both activates a range of haematopoietic cells and enhances their survival (Strainic, Liu et al. 2008; Markiewski, DeAngelis et al. 2009), it might also bind to leukaemia cells, promoting their growth. The actions of C3a might also not be solely mediated through C3aR as C3a has also been shown to signal through the alternative C5a receptor C5aR2 (Boos, Campbell et al. 2004; Boos, Szalai et al. 2005). This theory could be explored by assessing plasma C3a levels in tumour bearing WT and C3aR-/- mice and determining whether C3aR and/or C5aR2 are expressed on C1498 cells *in vitro* and *in vivo*. C3aR antagonists and C5aR2 knock-out mice could subsequently be used alongside C1498 cells, genetically modified to silence receptor expression, to test the hypothesis. This series of experiments could identify a novel mechanism by which complement promotes tumour growth.

4.3.4.3 MAC

Whilst complement is believed by some to facilitate lysis of cancer cells (Rutkowski, Sughrue et al. 2010), MAC has never been demonstrated to clear malignant cells and hence protect against the development of tumours *in vivo*. Earlier in this thesis I discussed the potential role for sub-lytic MAC in protecting malignant cells from immune clearance. In order to explore these opposing theories I used C6-/- mice, which are unable to form MAC, to investigate whether MAC affected the progression of AML. Increased AML progression would support a role for complement-mediated lysis in clearing malignant cells. A reduction in disease progression would support a role for complement-induced protection.

Studies demonstrated that C6-/- mice were partially protected against the progression of C1498 AML (Figure 4.28). This suggests that the ability to produce

MAC results in a favourable environment for the progression of AML. This supports the hypothesis that sub-lytic MAC on the surface of malignant cells might be protecting against elimination by the immune system *in vivo*.

Protection against AML was not observed in mice treated with C6 antisense oligonucleotide, despite its use achieving an almost 90% reduction in plasma C6 levels (Figures 4.29 and 4.30). As this reagent functions by preventing the production of C6 by the liver, it is possible that it is less effective at the tissue level which might result in inadequate impingement on local C6 production in the tumour microenvironment. Hence, sub-lytic levels of MAC could still be being produced in these C6 depleted (as opposed to fully deficient) animals. These data do not therefore weaken the case for complement-induced protection being an *in vivo* mechanism by which elimination of malignant cells is prevented.

Figure 4.34 summarises components of the complement system where impingement results in a significant survival effect in the IV C1498 cell model of AML.

4.3.5 Tumour distribution

During the course of the experiments described, I noted that mice treated with anti-C5 mAb, developed far more peripheral soft tissue masses (75%) than WT mice in whom isolated peripheral tumours were a rare event (approx 5%). Anti-C5 MAb treated mice were also noted to have a far lower incidence of liver and ovarian lesions (<40%) than WT controls (>90%). A review of tumour distribution in C6-/- mice revealed that they also had approximately half the incidence of liver masses than WT controls and twice as many soft tissue masses. WT mice do occasionally develop soft-tissue tumours but these are generally in addition to central tumours and even then occur at a far lower incidence than seen in mice that lack MAC.

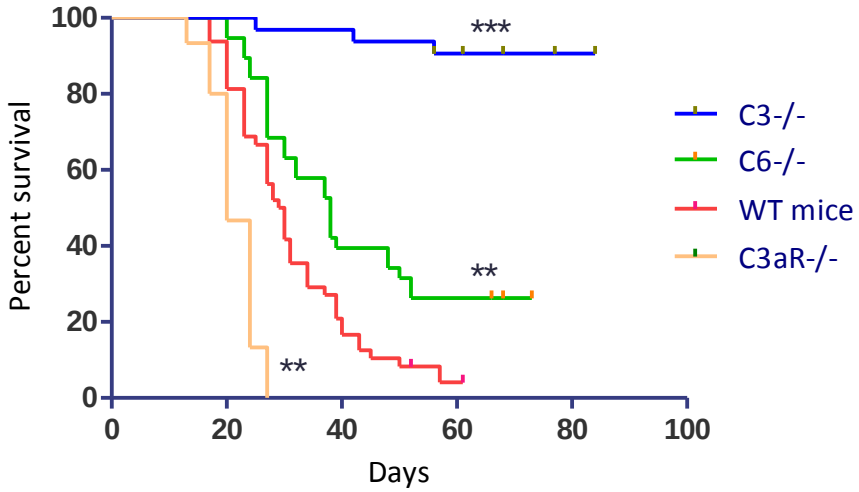


Figure 4.34: Summary of significant phenotypes. Following an IV inoculum of 5 x 10^6 C1498 murine AML failed to progress in the majority of C3-/- mice. C6-/- mice had a mildly protective phenotype resulting in approximately 25% long-term survivors. Contrary to this, AML progressed more rapidly in C3aR-/- mice.

In the absence of MAC, mice appear to be protected from the progression of AML at central sites, such as the liver and ovaries, generally the first places that tumours develop in WT mice, however mice then go on to develop tumours at peripheral sites. As complement is primarily produced in the liver, tumour distribution might reflect a gradient effect in local complement activity. Hence, the absence of MAC might facilitate the clearance of tumours from central sites but might fail to have an effect at peripheral sites where complement might have a less active role. Alternatively, peripheral tumours might simply be unusual in WT mice because AML progression in the livers and ovaries leads them to be killed before peripheral tumours become detectable.

The potential role for complement in influencing the location of leukaemic deposits led me to review the tumour distribution across all experiments. I noticed that no WT mice treated with C5aRA developed peripheral soft tissue masses. Whilst AML progressed in the liver and ovaries of C5aRA treated mice at a similar frequency to WT mice, those that failed to develop early lesions, did not go on to develop soft tissue tumours and became long-term survivors. Power calculations suggest that this finding may be statistically significant if more mice (approx. 10) were analysed.

C5a is crucial in order for granulocytes and monocytes to egress from the BM (Borkowska, Suszynska et al. 2014). C5aR stimulation might therefore also facilitate the movement of C1498 AML cells from the vasculature into peripheral tissues. However, the high incidence of soft-tissue masses observed in mice treated with anti-C5 mAb, who are unable to produce C5a, is contradictory to this hypothesis. Blocking the C5aR appears to results in less peripheral tumours whereas preventing production of C5a results in far more. This discrepancy led me to speculate as to whether an alternative mechanism other than signalling via the main C5aR could be involved. The key difference between mice unable to produce C5a and treated with the C5aRA would be their plasma levels of C5a, leading me to postulate that the phenotype could be the result of C5a signalling via an alternative receptor.

Whilst the vast majority of C5a's effects are thought to be mediated through C5aR, a second C5a receptor, C5aR2 (formerly known as C5L2), also exists, the role of which is rather contentious. C5aR2 is often referred to as a 'decoy' receptor which, by binding and removing active complement fragments from the extracellular environment, suppresses the inflammatory nature of C5aR stimulation (Scola, Johswich et al. 2009). However other studies have suggested that C5aR2 has a functional role in sepsis (Rittirsch, Flierl et al. 2008) and in inflammation (Huber-Lang, Sarma et al. 2005). The reduction in peripheral tumours observed in C5aRA treated mice could thus not be due to the absence of signalling via the C5aR but to an increased level of signalling via C5aR2.

Experiments designed to look at tumour distribution are required in order to further investigate my findings. An exploration of signalling via the C5aR2 in my model and its effect on tumour distribution might then also be warranted. C5a levels in plasma from tumour bearing mice will indicate whether there is any clear correlation with tumour distribution. Analysis of the expression of C5aR and C5aR2 on various murine tissue types including vasculature, liver and soft tissue along with the C1498 AML cells themselves would also be informative.

It should be noted that all statistically significant survival advantages observed herein have been seen in genetically modified animals. This may be due to the need for complete ablation as opposed to blockade of complement components or to the tissue penetration of reagents which may not be sufficiently thereby limiting their efficacy at tumour sites. It could also suggest that my findings are the result of inconsistencies with the genetically modified mouse model system. Whilst genetically modified mice are powerful tools they have significant limitations. The mouse and human genomes are extremely similar- 97.5% of functional human genes are conserved in the mouse (Mural, Adams et al. 2002). However, even small genetic disparities, in combination with subtle differences in function between human and mouse versions of the same gene, can translate into major distinctions. Modification of a given gene does not always result in the predicted phenotype with outcome also being influenced by epigenetic and environmental factors (Tarantino,

Sullivan et al. 2011). This results in significant inter-strain variation in the phenotype of mice with the same genome making interpretation of results from studies of genetically modified mice more challenging. Unless findings are extremely robust, results described in mice have proved extremely unlikely to translate into humans (Mak, Evaniew et al. 2014).

The restriction of my findings to genetically modified animals, whether due to incomplete efficacy of the therapeutic agents explored or limitations in the genetic models used, has implications on the potential for complement therapeutics to impact on human disease.

4.4 Concluding remarks

I*n vivo* studies using a C1498 murine model of AML have resulted in several exciting discoveries. I have discussed potential mechanisms that could underpin these findings including complement-induced protection, C3a mediated stimulation of tumour cells, iC3b mediated tumour seeding and migration and C5a mediated metastasis. Further studies are warranted in order to fully characterise the role of complement in mouse AML and the relevance of these findings to human disease.

Chapter 5: Final Discussion

The work presented in this thesis explored the role of complement in the setting of AML. It investigated both complement-induced protection *in vitro* and the role of complement in the progression of AML *in vivo.*

In Chapter 3, I explored whether sub-lytic complement attack protected against NK cell lysis *in vitro* then went on to explore the genetic signature of complement-induced protection. Results of these studies demonstrated that whilst sub-lytic complement offers convincing protection against a subsequent lytic complement attack, no convincing protection against NK cell killing is seen. Despite a strongly protective phenotype, no genetic signature for complement-induced protection was observed. This was despite the ability to identify a genetic signature for other experimental variables such as exposure to serum. This strongly suggests that an alternative mechanism such as actin re-modelling or depletion of lipid rafts could be involved.

In Chapter 4, I investigated the role of the complement system on the progression of AML *in vivo.* A combination of mice deficient in various complement components and complement inhibitors were used to study the role of various complement components in AML. The role of three complement-mediated processes and their underlying mechanisms were explored in the context of AML. These were:

i) C5a and C3a anaphylatoxins,
ii) Complement-induced protection and iii)
iii) iC3b mediated cell adherence and migration,

Key Finding	Potential Implication
AML fails to progress in C3-/- mice.	The complement system is critical to the progression of AML.
The protective effect in C3-/- mice is overcome by $CD8^+$ and to a lesser extent $CD4^+$ T cell depletion.	Complement impinges on the efficacy of CTLs in AML.
AML cells administered SC to C3-/- mice grow at the same rate as in WT mice.	Complement is critical to seeding of AML cells.
Administering anti-C5 mAb or C5aRA to WT mice had no impact on survival	Impinging on C5a had no impact on progression of AML
AML progressed more rapidly in C3aR-/- mice.	Either i) C3aR signalling protects against AML or ii) C3a signalling via C3aR expressed by AML promotes progression.
C6-/- mice had reduced progression of AML.	Complement-induced protection could be an *in vivo* phenomenon.
C6-/- and WT mice treated with anti-C5 mAb had fewer central tumours (ns).	MAC promotes progression of AML at central sites
WT mice treated with C5aRA had fewer peripheral tumours (ns).	C5a signalling promotes progression of AML at peripheral sites

Table 5.1: Key Findings of *In Vivo* Studies of the Role of Complement in the Progression of AML and their Potential Implications.

5.1: Complement-induced protection *in vitro*

5.1.1: Sub-lytic complement offers no convincing protection against NK cell killing

Despite investigating multiple variations to my experimental protocol including timescale analysis, altering effector:target (E:T) ratio, using alternative PBMC donors and developing an alternative combination assay, no convincing evidence of *in vitro* complement-induced protection from NK cell attack was observed during

my studies. This could be the result of the extreme susceptibility of K562 cells to NK mediated lysis meaning that sub-lytic complement attack is not sufficiently protective in this setting. As K562 was used in the original studies describing CIP and is widely used in the study of NK biology, it was the obvious choice of cell line to be used in these studies. However, a cell line less susceptible to NK cell lysis might offer more insight into the role of CIP in this setting.

Previous studies used purified perforin to demonstrate a cross-protective effect between sub-lytic complement and other pore-formers (Reiter, Ciobotariu et al. 1995). In contrast to complement, which relies purely on MAC to disrupt cell homeostasis and lyse cells, NK cells mediate cytotoxicity via a range of distinct mechanisms. Whilst the most studied mechanism is via perforin mediated degranulation, this is reliant on the formation of an immunological synapse which is the result of a complex interaction between activating cell surface receptors counteracted by signals from inhibitory receptors, most of which bind to MHC class 1 molecules (Bryceson and Long 2008). A sub-lytic complement attack will result in cellular stress which can lead cells to down-regulate MHC class 1 and up-regulate ligands for NK activating receptors, thus making cells more susceptible to NK attack (reviewed in (Chan, Smyth et al. 2014)). This stress response could compensate for protection provided by CIP against the insertion of perforin into the cell membrane. Increased numbers of activated NK cells forming immunological synapses could compensate for a hampered ability to form cytolytic pores thus explaining why CIP was not observed in a whole cell assay.

NK cells degranulation releases both pore-forming perforin and granzyme which enters target cells via the pore and induces apoptosis. The presence of granzyme in the whole cell assay, which might be able to enter sub-lytically attacked cells, might trigger apoptosis independently of direct cell lysis. In addition to this, NK cells also exert their cytotoxic effects on target cells through death receptor pathways such as TRAIL and Fas which induce apoptosis independently of NK cell degranulation. Thus the whole cell assay offers a far more complex environment which is likely to be why a protective effect was not observed.

5.1.2: Complement-induced protection does not have a unique genetic signature

A comprehensive microarray analysis revealed no significant gene expression changes an unusual finding in this setting. The strong 'protected' phenotype was confirmed in each test condition included in the analysis. This, along with our ability to clearly observe a 'serum effect', implies that if the protected phenotype was due to alterations in gene transcription, it would have been detected. I therefore concluded that CIP was dependent on an alternative mechanism.

The insertion or shedding of membrane attack complexes could result in structural or compositional change to the cell membrane, or underlying scaffolding elements, preventing MAC or other pore-formers from being inserted into the cell membrane. Mechanisms such as the preferential loss of cholesterol-rich regions of the cell plasma membrane, termed lipid rafts, could impinge on subsequent attack. If MAC is preferentially inserted into these lipid rich areas, a sub-lytic complement attack would not only lead to shedding of membrane vesicles containing MAC, but could also result in the loss of lipid rafts. Loss of these cholesterol rich areas could thus impinge on the ability of MAC or other pore-formers to insert into the cell membrane until lipid rich areas are replenished.

An alternative mechanism for CIP could be re-configuration of the actin skeleton, a mechanism utilised by pathogens such as cytomegalovirus (CMV). Despite a clear role for actin reconfiguration by cytotoxic cells in the development of the immune synapse, there is surprisingly little data regarding the role of the actin cytoskeleton in target cells. Their ability to reorganise actin could affect the ability of pore-formers to insert into the cell membrane. This could therefore be a potential mechanism through which sub-lytic complement attack results in resistance from further pore-forming attack.

5.2: Complement is critical to the progression of AML

The over-reaching effects of the complement system were investigated using C3-/- mice which lack the ability to produce MAC, C3a and C3b. C3-/- mice were resistant to progression of AML with 29/32 failing to develop detectable disease (P <0.0001***) (Figure 4.7).

5.2.1: Complement impinges on the efficacy of CTLs in AML

T cell depletion studies suggest that C3-/- mice are able to clear AML through a CD8+ T cell mediated mechanism (Figure 4.14). However, C3-/- mice do not appear to mount a superior memory T cell response as measured by IFN-γ response to exposure to irradiated tumour cells following vaccination (Figure 4.16).

The dose of C1498 used in these studies was sufficient to trigger an adaptive immune response, with even WT mice having more aggressive disease following $CD8^+$ T cell depletion (P<0.0001, n= 8, Figure 4.13). The CTL response, whilst ineffective at clearing AML in a complement sufficient environment, successfully prevented the progression of AML in a complement deficient environment. Injection of the tumour cells SC also induced a visible immune response in both C3-/- and WT mice. Yet, whilst the tumours in the majority of WT mice escaped from this immune control, C3-/- mice successfully cleared tumours and remained disease free (Figure 4.32). This supports a role for complement impinging on the efficacy of primary $CD8^+$ T cell response and leading to immune escape.

ELISpot analysis of IFN-γ responses generated following injection of mice with irradiated tumour cells however, demonstrated that memory T cell responses in C3-/- mice were not superior to those observed in WT mice (Figure 4.16). As the number of splenocytes was standardised in these assays, it is conceivable that C3-/- mice produce higher numbers rather than more effective CD8+ T cells thus accounting for the more effective immune response observed *in vivo*.

It is also the case that Fluorospot assays, which use multiple fluorescent labelled anti-cytokine detection antibodies to allow measurements of multiple cytokines or granzymes might offer a better measure of CTL response.

Observed effects could also be the result of critical differences in immune responses generated by vaccination with apoptotic (irradiated) vs inoculation with live cells. *In vivo* exposure to live cells leads to more effective cross-presentation by DCs resulting in more effective CTL responses than observed following immunization with irradiated cells (Matheoud, Perie et al. 2010; Volovitz, Marmor et al. 2011). Thus, exposure to irradiated cells may fail to trigger the full CTL response observed in C3-/- mice following exposure to live inoculums of leukaemia.

It is also possible that complement may selectively impinge on primary CTL responses whilst not having a role in the generation of a memory response. *In vitro* generated CTLs often fail to control established AML *in vivo* (Zhou, Bucher et al. 2009). This could be addressed by investigating the efficacy of CTLs generated in a complement sufficient vs deficient environment when reinfused into complement–deficient and sufficient CD8-depleted mice prior to exposure to live AML cells.

This would help to address whether memory T cell responses against AML are different when raised in a complement deficient vs sufficient environment *in vivo* and might also reveal whether complement has a selective effect on primary T cell responses. If CTL therapy is both ineffective and is impinging on a more robust primary response in C3-/- mice, then increased progression of AML might be observed in C3-/- mice following vaccination.

Uncontrolled complement activation, mediated by fH suppression by PTX53, is an essential component of tumour-promoting inflammation (Bonavita, Gentile et al. 2015). Tregs alter their phenotype depending on the concomitant inflammatory response (Yamaguchi, Wing et al. 2011). Complement can impinge on immune responses via induction of Treg responses with MCP stimulation steering human CD4+ T cells towards an immunosuppressive role (Kemper, Chan et al. 2003), a

finding also supported by the low numbers of Tregs observed in human C3 deficiency (Ghannam, Pernollet et al. 2008). Transplant tolerance is also dependent on C3, with FOXP3 + Tregs failing to suppress the immune response in the absence of complement (Sacks and Zhou 2012). Progression of C1498 AML leads to an accumulation of regulatory T-cells (Treg) at disease sites (Zhou, Bucher et al. 2009). Treg depletion alone is ineffective at clearing tumours, requiring concurrent transfer of anti-AML CTLs (Zhou, Bucher et al. 2009). It therefore perhaps most likely that complement is impinging on adequate immune responses to AML via the induction and/or activation of Tregs. Thus combining Treg- and complement-depletion might prove an effective way of optimising both natural and induced CTL responses.

The C1498 model of AML has also been shown to induce MDSCs. Interestingly, whilst these are both increased in numbers and able to suppress the activity of CD4+ T cells, they do not appear to suppress the activity of $CD8^+$ T cells (Gibbins, Ancelet et al. 2014). This could explain why neutralising C5a, an intervention that resulted in MDSC mediated suppression of $CD8^+$ T cell responses in other disease models, failed to prevent progression in this model.

The SC model offers an excellent opportunity to explore the mechanism of immune escape, observed in WT but not C3-/- mice. Immunohistochemistry, Fluorospot assays and immunophenotyping could be used to compare kinetics, quality and magnitude of T-cell responses in complement sufficient versus deficient mice. Adoptive transfer of cells identified to play a key role, between C3-/- and WT mice would confirm key mechanisms at play.

5.2.2: Complement is critical to seeding of AML

In order to infiltrate tissues, tumour cells must bind to and activate platelets and leukocytes allowing them to adhere to vessel walls and extravasate (Gay and Felding-Habermann 2011). Complement activation results in iC3b binding to CR3 (CD11b/CD18) which is essential for tethering between activated platelets and

polymorphonuclear leukocytes (PMN) (Hamad, Mitroulis et al. 2015). As a malignant form of PMN, C1498 also expresses CD11b (Barrett and Jiang 2000), CR3 expression on the surface of leukaemia cells could thus be critical to its ability to seed. Alternatively iC3b deposition on malignant cells might result in its tethering to the platelet/PMN complex. In a complement deficient environment, the absence of iC3b might thus prevent tethering between C1498 and platelets or a platelet/ PMN complex, preventing them from adhering to vessel walls and migrating into tissues. We therefore postulated that the lack of progression observed in complement deficient mice, when leukaemia was administered IV, was secondary to this inability to tether and migrate.

When C1498 cells were administered SC, thus removing the need for cells to adhere and seed, comparable tumour growth was observed in C3-/- and WT mice (Figure 4.32). This supports the hypothesis that complement is required in order for C1498 AML cells to seed from blood vessels into tissue.

The prolonged presence of leukaemia cells within the circulation, following IV injection, might provide an antigen load capable of triggering a more robust immune response in C3-/- mice. An inability to seed might also starve cells of pro-survival signals, provided by a tumour niche, essential to cell survival and proliferation. Thus cell death could be independent of immune response making the role of the immune system secondary to the influence of complement on tumour take.

AML-niche interactions have a key role in development, progression, chemotherapy resistance and relapse in AML (Rashidi and DiPersio 2016). Myeloid blasts express a range of adherence receptors including CXCR4 and the ES ligand modulate adherence, migration, survival and proliferation (Peled, Kollet et al. 2000; Winkler, Barbier et al. 2012; Chien 2013; Kremer, Dudakovic et al. 2014). A role for complement in mediating the adherence and migration of leukaemia cells would thus be of critical importance.

Identification and quantification of leukaemia cells in the circulation following IV injection might support the seeding hypothesis. Staining for platelets bound to the surface of circulating AML cells following IV administration to WT vs C3-/- mice would clarify whether platelets play a key role. Anti-platelet agents such as clopidogrel could be used to confirm the role of platelet aggregation on tumour cell take following IV administration. Small molecule inhibitors of adherence molecules such as E-selectin (GMI-1271) could also be utilised in my studies (Chien 2013).

Although I demonstrated no evidence that C1498 cells produce C3 *in vitro* or *in vivo*, it remains important to address the possibility that C3 (whilst at undetectable levels) acts as an immunogen in C3-/- mice. Reconstituting the complement system in C3-/- mice using purified C3 would confirm that the presence of C3 is critical to AML progression. C3 reconstitution at a range of time points in the IV model could be used to explore the relative importance of C3 on tumour cell seeding, induction of both CTL and Treg responses. Transient complement depletion with CVF could also be used to explore my findings in WT mice. This would also allow me to explore the role of NK cells on AML progression in a complement deficient environment as NK cells could be depleted in complement sufficient mice ahead of decomplementation with CVF.

5.2.3 C5a has no impact on progression of AML

Results fail to demonstrate any impact on survival in mice treated with either anti-C5 antibody or C5aRA (Figures 4.21 and 4.23) despite other reports indicating that C5a is the most widely cited complement component thought to play a role in promotion of tumour growth (Wang, Veeramani et al. 2009; Corrales, Ajona et al. 2012; Gunn, Ding et al. 2012; Nunez-Cruz, Gimotty et al. 2012; Janelle, Langlois et al. 2014; Vadrevu, Chintala et al. 2014; Downs-Canner, Magge et al. 2015; Imamura, Yamamoto-Ibusuki et al. 2015). Whilst this is most widely attributed to C5a promoting the development of cancer by suppressing the anti-tumour CD8+ T cell-mediated response via the recruitment and augmentation of MDSCs (Markiewski, DeAngelis et al. 2008; Corrales, Ajona et al. 2012; Vadrevu, Chintala et al. 2014), C5a

has also been shown to support tumour progression by promoting angiogenesis (Corrales, Ajona et al. 2012; Nunez-Cruz, Gimotty et al. 2012). Some studies suggest that the level of C5a expression might be critical to the malignant phenotype (Kim, Martin et al. 2005; Gunn, Ding et al. 2012). C5a might also be particularly important in the setting of metastasis (Vadrevu, Chintala et al. 2014; Imamura, Yamamoto-Ibusuki et al. 2015).

As discussed earlier, whilst MDSCs are increased in leukaemic animals and suppress the activity of CD4+ T cells, they did not appear to have a suppressive effect on CD8+ T cells (Gibbins, Ancelet et al. 2014). The protective effect of impinging on C5aR in other disease models is often mediated via suppressing MDSC activity against CD8+ T cells. If MDSCs present in leukaemic animals are truly unable to suppress CD8+ T cell activity, this mechanism might be irrelevant in this setting.

The lack of observed effect observed could simply be due to the aggressive phenotype of C1498. This is the explanation offered by other groups for disparities observed between tumour models (Markiewski, DeAngelis et al. 2008; Vadrevu, Chintala et al. 2014). The rapid tumour growth observed following the administration of C1498 leukaemia IV could therefore be beyond the capacity of any boost to CD8 function achieved by C5a inhibition. An exploration of the efficacy of the C5aRA in the setting of C1498 cells administered via a SC route, which might prompt a more efficient CD8+ T cell response, would be informative.

The lack of survival advantage observed in mice treated with anti-C5 mAb or C5aRA might be due to failure to completely abrogate local production of C5a or block C5aR at the tumour site.

Despite multiple studies supporting a role for C5a in tumour progression, this is still controversial as C5a over-expression in tumour cells has also resulted in tumour regression in a mouse model of breast cancer (Kim, Martin et al. 2005). Translational research also supports a positive role for C5a in anti-tumour monoclonal antibody therapy (Allendorf, Yan et al. 2005; Li, Allendorf et al. 2007;

Fuenmayor, Perez-Vazquez et al. 2010). Hence the role for C5a in tumour progression remains controversial.

It is also the case that the profoundly protective phenotype observed in C3-/- mice is unlikely to be mediated via C5a, as C3-/- mice are widely accepted to produce C5a. Even in the absence of complement generated C5 convertase, C5 can be cleaved into its active forms by a range of other proteolytic enzymes, such as thrombin and plasmin (Huber-Lang, Sarma et al. 2006; Borkowska, Suszynska et al. 2014). With this in mind, it is unlikely that C5a is completely absent in C3-/- mice, further supporting the conclusion that C5a does not play a key role in the progression of AML in the model described herein.

5.2.4: C3a has a role in AML progression

C3aR-/- mice were used to explore the role of C3a-C3aR signalling on progression of AML. Somewhat surprisingly, AML progressed more rapidly in mice lacking a C3a receptor (Figure 4.25). This rapid progression of AML observed in C3aR-/- mice might suggest a role for C3a signalling in limiting disease progression; a finding somewhat at odds with the protective phenotype observed in C3-/- mice, in which C3a signalling would also be absent.

A protective role for C3a could be explained by the fact that, in addition to its pro-inflammatory role, C3a also has anti-inflammatory properties (Coulthard and Woodruff 2015). Thus, in the settings of both sepsis and an acute inflammatory response C3a can act in direct opposition to C5a (Hollmann, Mueller-Ortiz et al. 2008; Wu, Brennan et al. 2013). It is therefore feasible that C3a could have a protective role in the setting of malignancy.

This is challenged by other studies which have conversely demonstrated a role for C3a-C3aR signalling in promoting tumour growth (Guglietta, Chiavelli et al. 2016; Nabizadeh, Manthey et al. 2016). Using a B16 model of melanoma, C3aR-/- mice exhibited retarded tumour growth and treatment with a C3aR antagonist (C3aRA)

inhibited growth of established tumours (Nabizadeh, Manthey et al. 2016). C3aR deficiency/ inhibition affected tumour-infiltrating leukocyte populations leading to increased neutrophil and $CD4^+$ T cell infiltration but reduced infiltration by macrophages (Nabizadeh, Manthey et al. 2016). Neutrophil depletion reversed the tumour inhibitory effects observed in C3aR-/- mice (Nabizadeh, Manthey et al. 2016). C3aR deficiency/ inhibition also resulted in reduced tumour growth in murine models of $BRAF^{V600E}$ mutant melanoma, colon and breast cancer (Nabizadeh, Manthey et al. 2016). In the second study, using an $APC^{min/+}$ model of colorectal cancer, tumourigenesis resulted in an upregulation of C3aR on neutrophils which led to activation of the complement cascade and promoted tumour progression. Tumour suppression observed in $APC^{min/+}$/C3aR-/- mice, was mediated via a reduction in pro-tumorigenic neutrophils (Guglietta, Chiavelli et al. 2016). The fact that both of these studies found C3a's role to be mediated via neutrophils might explain the disparate effects observed in the setting of leukaemia, where neutropenia is a key feature.

Another possibility is that C3a does not actually impede the progression of AML but that the observed phenotype is the result of C3a signalling via receptors on the malignant cells. C3a levels are elevated in C3aR-/- mice, due to absence of the receptor. As C3a both activates a range of haematopoietic cells and enhances their survival (Strainic, Liu et al. 2008; Markiewski, DeAngelis et al. 2009), it might also bind to leukaemia cells, promoting their growth. The actions of C3a might also not be solely mediated through C3aR as C3a can also signal through the alternative C5a receptor C5aR2 (Boos, Campbell et al. 2004; Boos, Szalai et al. 2005). This theory has the advantage of also partially explaining the phenotype observed in C3-/- mice which are unable to produce C3a and would thus be unable to promote leukaemic growth.

This could be further explored by assessing plasma C3a levels in tumour bearing WT and C3aR-/- mice, determining whether C3aR and/or C5aR2 are expressed on C1498 cells *in vitro* and *in vivo* and exploring whether C3a influenced proliferation of C1498 *in vitro*. C3aR antagonists, C3aR-/- and C5aR2-/- mice could subsequently be

investigated in combination with C1498 cells, genetically modified to silence receptor expression, in order to test the hypothesis. This series of experiments could identify a novel mechanism by which complement promotes tumour growth.

5.2.5: Complement-induced protection could be an *in vivo* phenomenon

C6-/- mice, which are unable to form MAC, were used to investigate whether the presence of MAC affected the progression of AML. In the absence of MAC, mice were protected against the progression of C1498 AML (Figure 4.28). The presence of MAC resulted in a favourable environment for the progression of AML, thus supporting the hypothesis that sub-lytic MAC protects malignant cells against elimination by the immune system *in vivo*. The observed effect was however insufficient to explain the almost complete protection observed in C3-/- mice.

The 90% reduction in C6 levels achieved by treatment with C6 antisense oligonucleotide did not result in any survival advantage. This is perhaps not surprising as this incomplete ablation might result in a sub-lytic level of MAC being produced in the tumour microenvironment. These data do not therefore weaken the case for complement-induced protection being an *in vivo* mechanism by which elimination of malignant cells is prevented.

It is concerning that all statistically significant survival advantages observed in these studies are observed in genetically modified animals. Although this may be due to an inability of reagents to result in completely ablation at the tissue level it might also suggest that my findings are the result of inconsistencies with the genetically modified mouse model system. Whilst genetically modified mice are powerful tools they have significant limitations. Whilst mouse and human genomes are extremely similar, even small genetic disparities, in combination with subtle differences in function, can translate into major distinctions. Modification of a given gene does not always result in the predicted phenotype with outcome also being influenced by epigenetic and environmental factors (Tarantino, Sullivan et al. 2011). Unless

findings are extremely robust, results described in mice have proved extremely unlikely to translate into humans (Mak, Evaniew et al. 2014).

It is perhaps more concerning that the protective effect observed in the C6-/- mice was not seen in mice treated with the anti-C5 mAb, who were also unable to form MAC. Despite no observed survival advantages I did note some profound differences in tumour location in the anti-C5 mAb treated mice which led me to explore the effect of complement on tumour distribution.

5.2.6: Complement has a potential role in tumour distribution

As studies performed in this thesis were not performed in order to explore an effect of complement on tumour distribution the observations made regarding this are provisional, but none-the less interesting. The observation that mice treated with anti-C5 mAb had an extremely high incidence (75%) of peripheral tumours led me to review tumour distribution across my studies. This led me to observe that mice lacking MAC, either due to genetic knock-out (C6-/-) or administration of anti-C5 mAb developed fewer central (liver, ovary) tumours but more peripheral (limbs or back) tumours. Central sites were generally the earliest sites of disease progression with mice developing peripheral tumours at a later stage. Complement is primarily produced in the liver, meaning there could be a gradient of complement activation. The absence of MAC might thus facilitate the clearance of tumours from central sites but fail to have an effect at peripheral sites where complement might have a less active role. Peripheral tumours might only be unusual in WT mice as AML progression in the livers and ovaries leads them to be killed before peripheral tumours become detectable.

It was also noted that no WT mice treated with C5aRA developed peripheral tumours. C5aRA treated mice had a similar frequency of central tumours however, those that failed to develop early lesions, did not go on to develop peripheral tumours and became long-term survivors. Power calculations suggest that this finding may be statistically significant if additional mice (approx. 10) were analysed.

It is notable that blocking the C5aR appears to results in less peripheral tumours whereas preventing production of C5a results in far more. The key difference between mice unable to produce C5a and those treated with the C5aRA would be their plasma levels of C5a, leading me to postulate that the phenotype could be the result of C5a signalling via an alternative receptor.

Whilst the vast majority of the effects of C5a are thought to mediated through C5aR, a second C5a receptor, C5aR2, exists, the role of which is rather contentious. Whilst previously discounted as being just a 'decoy' receptor, other studies have suggested that C5aR2 has a functional role in sepsis (Rittirsch, Flierl et al. 2008) and in inflammation (Huber-Lang, Sarma et al. 2005). The reduction in peripheral tumours observed in C5aRA treated mice could thus not be due to the absence of signalling via the C5aR but to an increased level of signalling via C5aR2.

These observations, though interesting, are provisional and additional experiments designed to look at tumour distribution, are required in order to further investigate my findings. This might lead to further studies such as whether plasma C5a levels correlate with tumour distribution, if signalling via C5aR2 affects tumour distribution and whether C5aR and C5aR2 are expressed by various murine tissue types along with the C1498 AML cells themselves.

5.3 Implications to AML patients

The restriction of my findings to genetically modified animals, whether due to incomplete efficacy of the therapeutic agents explored or limitations in the genetic models used, has implications on the potential for complement therapeutics to impact on human disease.

A major limitation of the mouse studies is the lack of a C3-specific inhibitor which would clearly be extremely useful for the studies described herein. Such an inhibitor, namely Compstatin, is however available for targeting of human C3.

Compstatin is a cyclic peptide which functions by binding C3 and interfering with C3 cleavage (Mastellos, Yancopoulou et al. 2015). By targeting C3, the hub of the complement cascade, this offers a more encouraging method of manipulating complement in the human disease setting and has shown promise in a range of immune and inflammatory disorders (Ricklin and Lambris 2013). Compstatin has demonstrated unparalleled efficacy at improving survival and preventing end organ damage in a model of severe sepsis, even when administered at a late stage (Silasi-Mansat, Zhu et al. 2010). Compstatin analogues have also proved effective in haemodialysis- induced inflammation (Reis, DeAngelis et al. 2015).

The anti-C5 monoclonal antibody Eculizumab limits acute graft rejection and prolongs graft survival and function in a proportion of renal and pancreatic transplant recipients (Biglarnia, Nilsson et al. 2011; Stegall, Diwan et al. 2011). Compstatin is currently under investigation in this setting in an attempt to achieve more consistent responses (Mastellos, Yancopoulou et al. 2015). Eculizumab is also used in the clinical management of paroxysmal nocturnal haemoglobinuria (PNH) in order to prevent complement-dependent intravascular haemolysis (Hillmen, Young et al. 2006). Responses are however variable due to amplification of C3 activation and persistent opsonisation of erythroctyes resulting in C3-mediated extravascular haemolysis in some patients (Hill, Rother et al. 2010; Risitano, Notaro et al. 2010). The strong therapeutic potential of direct C3 inhibition in PNH has been endorsed by both the EMA and US FDA recently granting orphan drug designation to the Compstatin derivative AMY-101 (Mastellos, Yancopoulou et al. 2015).

The complement system remains largely unstudied in the context of human AML. Historic studies demonstrated that complement was upregulated in active disease (Minh, Czink et al. 1983). Interestingly, high CD11b expression predicts poor prognosis in human AML (reviewed in (Xu, Li et al. 2015)) suggesting that the role of complement in tumour cell adhesion could be relevant to patients with AML. More recently, C3f-desArg has been identified as having a potential role in the AML diagnosis and MRD assessment (Liang, Wang et al. 2010). C3f derivatives have also

been identified in other forms of cancer (Villanueva, Martorella et al. 2006; Profumo, Mangerini et al. 2013).

C3f is formed when C3b is converted into iC3b by fI and fH and is rapidly degraded into C3fdesArg (Liang, Wang et al. 2010) (Figure 5.1). FH has a significant role in a range of malignancies (Junnikkala, Jokiranta et al. 2000; Raitanen, Marttila et al. 2001; Junnikkala, Hakulinen et al. 2002; Ajona, Castano et al. 2004; Wilczek, Rzepko et al. 2008).

Provisional transcriptome analyses of human myeloid blasts carried out in conjunction with our AML research group, identified complement dysregulation as being key to poor outcome in AML (Dr S. Coles, unpublished data). Further analysis, undertaken by myself and Dr Coles, revealed high expression of fH to be associated with poor prognosis (Figure 5.2). This finding was confirmed using a German AML diagnostic dataset available online (Metzeler, Hummel et al. 2008). FH was also identified as one of the most discriminatory genes by an independent group who defined a prognostic gene signature in AML (Bullinger, Dohner et al. 2008).

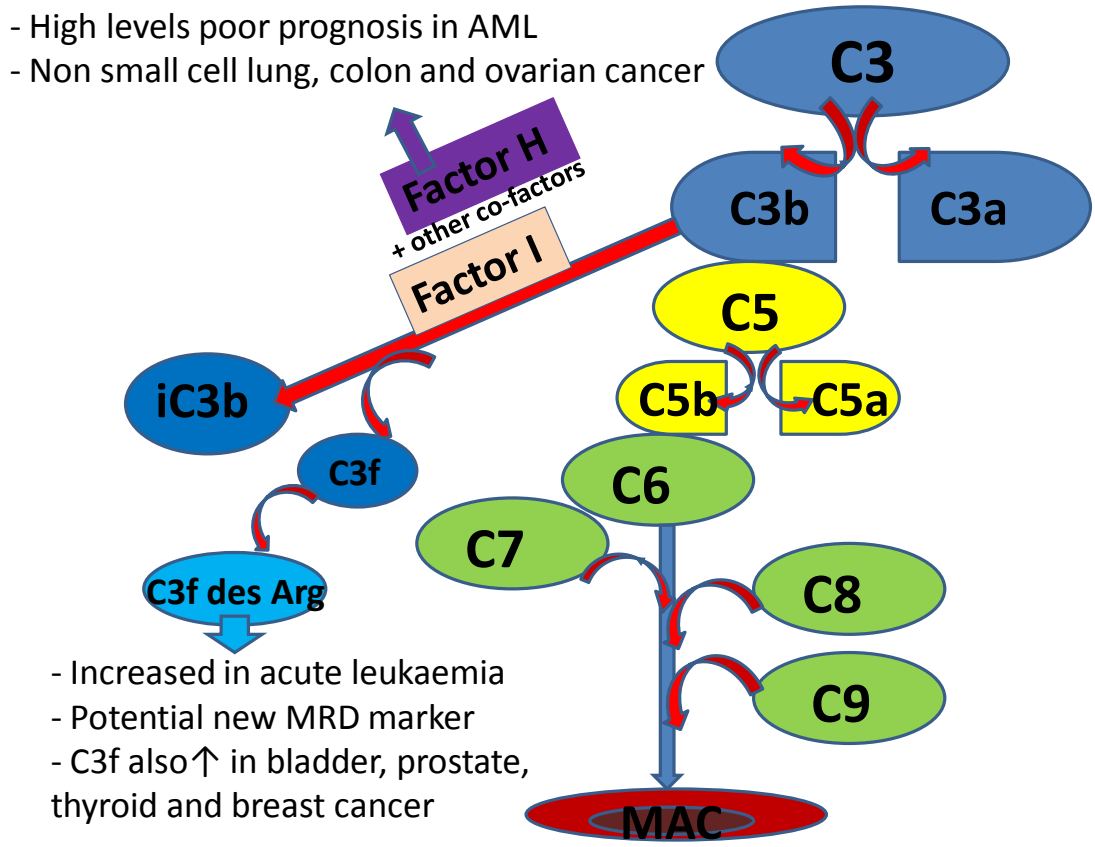


Figure 5.1: C3fdesArg and fH in cancer. C3b inactivation by fI and fH results in the release of C3f which is rapidly degraded into C3fdesArg. C3fdesArg has been identified as a potential new MRD marker in AML and is also elevated in a range of other malignancies. FH expression is also dysregulated in AML a finding also described in several other cancer types.

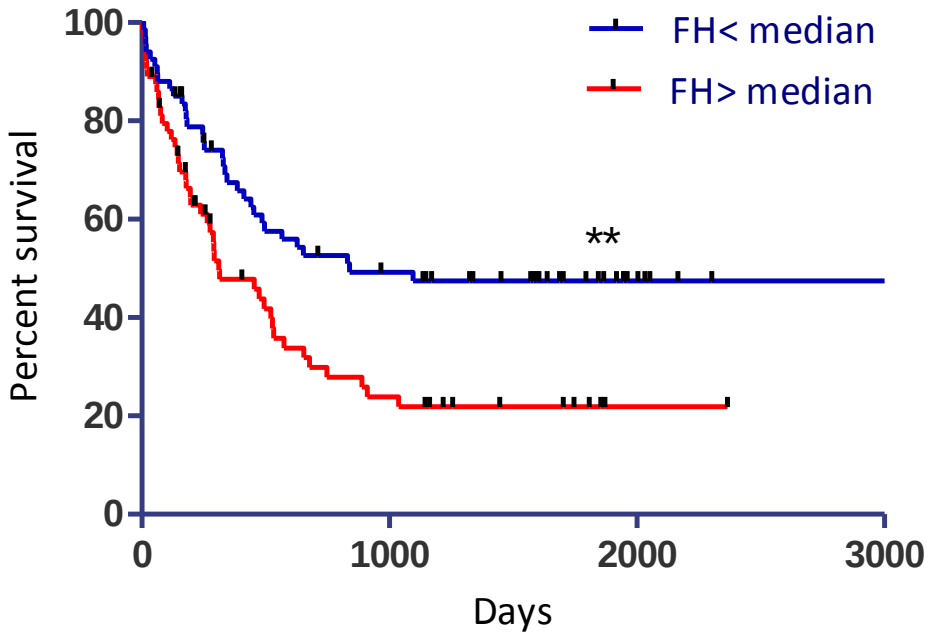
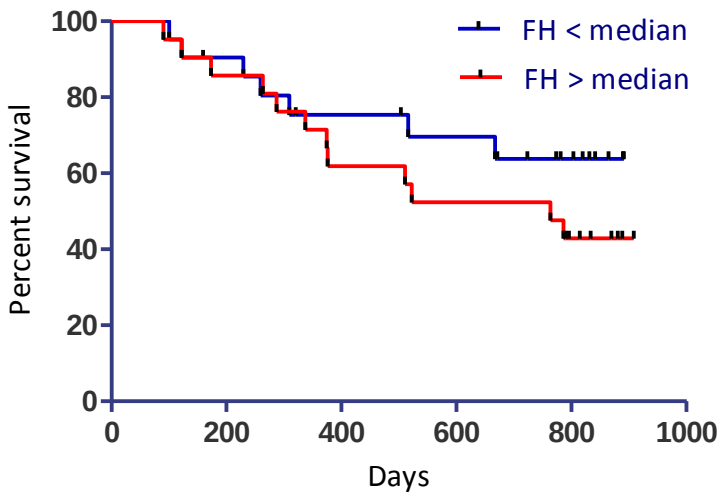


Figure 5.2: AML patient survival with respect to FH gene expression levels. Patient survival analysed with respect to FH gene expression levels > or < median. Kaplan Meier survival curve; P = 000041 (Log-rank (Mantel-cox) test), n= 131.

A.



B.

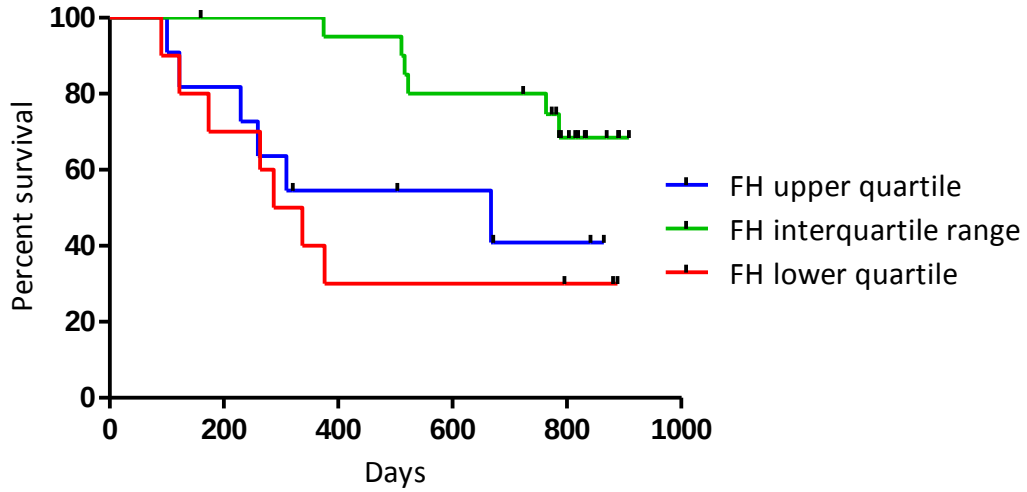


Figure 5.3: Dysregulation of Factor H levels in plasma from patients with AML. A. Provisional data reveals a trend for higher FH levels in plasma samples taken from AML patients at diagnosis conferring a worse prognosis (Kaplan Meier survival curve; P = 0.2914 (non-significant) (Log-rank (Mantel-cox) test), n = 42. B. Further analysis revealed that FH dysregulation rather than high or low level had a significant effect on survival. Patients with plasma FH levels in either the upper or lower quartile appear to have significantly worse survival than that observed in patients with a FH level within the inter-quartile range at diagnosis. Kaplan Meier survival curve; P = 00159 (Log-rank (Mantel-cox) test), n = 42.

Over the course of my studies, a bank of >1000 plasma and bone marrow supernatant samples from AML patients taken at diagnosis and post-treatment has been generated. Multiplexed complement protein ELISAs have been developed in our laboratory but were unavailable for use before the end of my studies. Provisional results generated using ELISAs to measure levels of fH, iC3b and TCC in diagnostic plasma samples from patients with AML support the microarray findings of high fH expression being associated with poor prognosis although larger numbers of samples need testing in order to determine whether these findings are significant (Figure 5.3). Interestingly provisional data suggest that fH dysregulation rather than simply a high or low level might be critical to outcome. Patients with plasma FH levels in either the upper or lower quartile have significantly worse survival than patients where the FH level at diagnosis lies within the inter-quartile range. P= 0.0159 n = 42 (Figure 5.3). This raises questions about the potential down-stream effects of FH expression as well as whether other factors, such as FH polymorphisms affecting function, might play a role. The multiplexed complement protein ELISAs are now available and could be used to comprehensively interrogate patient complotype in AML.

5.4: Concluding remarks

I*n vivo* studies using a C1498 murine model of AML have resulted in several exciting discoveries. I have discussed potential mechanisms that could underpin these findings including complement-induced protection, C3a mediated stimulation of tumour cells, iC3b mediated tumour seeding and migration and C5a mediated metastasis.

Immune therapies are already widely used in the treatment of AML, with novel agents constantly being investigated and developed for use. If complement has a role in the progression of AML, then complement-targeted therapeutics already available for use in humans, may not only have an independent role in improving spontaneous immune responses to leukaemia, but may also impact on all therapies aimed at controlling AML via immune mediated mechanisms.

References

(2013). "Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia." N Engl J Med **368**(22): 2059-2074.

(2015). "National cancer intelligence network cancer outcomes conference 2015, 8-10 june 2015, europa hotel, belfast." Eur J Cancer Care (Engl) **24 Suppl 1**: 1-82.

Abbas, A. K., C. Benoist, et al. (2013). "Regulatory T cells: recommendations to simplify the nomenclature." Nature Immunology **14**(4): 307-308.

Abdel-Magid, A. F. (2014). "Inhibitors of factor d may provide a treatment for age-related macular degeneration." ACS Med Chem Lett **5**(2): 106-107.

Acuto, O., V. Di Bartolo, et al. (2008). "Tailoring T-cell receptor signals by proximal negative feedback mechanisms." Nat Rev Immunol **8**(9): 699-712.

Adeegbe, D. O. and H. Nishikawa (2013). "Natural and induced T regulatory cells in cancer." Front Immunol **4**: 190.

Ajona, D., Z. Castano, et al. (2004). "Expression of complement factor H by lung cancer cells: effects on the activation of the alternative pathway of complement." Cancer Research **64**(17): 6310-6318.

Ajona, D., Y. F. Hsu, et al. (2007). "Down-regulation of human complement factor H sensitizes non-small cell lung cancer cells to complement attack and reduces in vivo tumor growth." Journal of Immunology **178**(9): 5991-5998.

Aksamit, R. R., W. Falk, et al. (1981). "Chemotaxis by mouse macrophage cell lines." J Immunol **126**(6): 2194-2199.

Alatrash, G., H. Jakher, et al. (2013). "Cancer immunotherapies, their safety and toxicity." Expert Opin Drug Saf **12**(5): 631-645.

Allavena, P., A. Sica, et al. (2008). "The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance." Immunol Rev **222**: 155-161.

Allendorf, D. J., J. Yan, et al. (2005). "C5a-mediated leukotriene B4-amplified neutrophil chemotaxis is essential in tumor immunotherapy facilitated by anti-tumor monoclonal antibody and beta-glucan." Journal of Immunology **174**(11): 7050-7056.

Almand, B., J. I. Clark, et al. (2001). "Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer." Journal of Immunology **166**(1): 678-689.

Alper, C. A., A. M. Johnson, et al. (1969). "Human C'3: evidence for the liver as the primary site of synthesis." Science **163**(3864): 286-288.

Andersen, M. H. (2014). "The targeting of immunosuppressive mechanisms in hematological malignancies." Leukemia **28**(9): 1784-1792.

Andoh, A., Y. Fujiyama, et al. (1998). "Detection of complement C3 and factor B gene expression in normal colorectal mucosa, adenomas and carcinomas." Clinical and Experimental Immunology **111**(3): 477-483.

Anguille, S., E. Lion, et al. (2011). "Interferon-alpha in acute myeloid leukemia: an old drug revisited." Leukemia **25**(5): 739-748.

Anguille, S., E. L. Smits, et al. (2014). "Clinical use of dendritic cells for cancer therapy." Lancet Oncol **15**(7): e257-267.

Anguille, S., V. F. Van Tendeloo, et al. (2012). "Leukemia-associated antigens and their relevance to the immunotherapy of acute myeloid leukemia." Leukemia **26**(10): 2186-2196.

Baatrup, G., N. Qvist, et al. (1994). "Activity and activation of the complement system in patients being operated on for cancer of the colon." Eur J Surg **160**(9): 503-510.

Badoual, C., S. Hans, et al. (2006). "Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers." Clin Cancer Res **12**(2): 465-472.

Baessler, T., J. E. Charton, et al. (2010). "CD137 ligand mediates opposite effects in human and mouse NK cells and impairs NK-cell reactivity against human acute myeloid leukemia cells." Blood **115**(15): 3058-3069.

Baessler, T., M. Krusch, et al. (2009). "Glucocorticoid-induced tumor necrosis factor receptor-related protein ligand subverts immunosurveillance of acute myeloid leukemia in humans." Cancer Research **69**(3): 1037-1045.

Balkwill, F. and A. Mantovani (2001). "Inflammation and cancer: back to Virchow?" Lancet **357**(9255): 539-545.

Baron, F., M. B. Maris, et al. (2005). "Graft-versus-tumor effects after allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning." J Clin Oncol **23**(9): 1993-2003.

Barrett, J. and Y.-Z. Jiang (2000). Allogeneic immunotherapy for malignant diseases. New York, Dekker.

Baruah, P., E. Simpson, et al. (2010). "Mice lacking C1q or C3 show accelerated rejection of minor H disparate skin grafts and resistance to induction of tolerance." European Journal of Immunology **40**(6): 1758-1767.

Battistelli, S., A. Vittoria, et al. (2005). "Protein S in cancer patients with non-metastatic solid tumours." Eur J Surg Oncol **31**(7): 798-802.

Bekisz, J., Y. Sato, et al. (2013). "Immunomodulatory effects of interferons in malignancies." J Interferon Cytokine Res **33**(4): 154-161.

Benekli, M., H. Baumann, et al. (2009). "Targeting signal transducer and activator of transcription signaling pathway in leukemias." J Clin Oncol **27**(26): 4422-4432.

Benekli, M., Z. Xia, et al. (2002). "Constitutive activity of signal transducer and activator of transcription 3 protein in acute myeloid leukemia blasts is associated with short disease-free survival." Blood **99**(1): 252-257.

Benzaquen, L. R., A. Nicholson-Weller, et al. (1994). "Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells." J Exp Med **179**(3): 985-992.

Berger, R., R. Rotem-Yehudar, et al. (2008). "Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies." Clin Cancer Res **14**(10): 3044-3051.

Bergmann, C., L. Strauss, et al. (2008). "T regulatory type 1 cells in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease." Clin Cancer Res **14**(12): 3706-3715.

Bergmann, L., D. K. Schui, et al. (1995). "The inhibition of lymphokine-activated killer cells in acute myeloblastic leukemia is mediated by transforming growth factor-beta 1." Exp Hematol **23**(14): 1574-1580.

Beurskens, F. J., M. A. Lindorfer, et al. (2012). "Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients." Journal of Immunology **188**(7): 3532-3541.

Beverley, P. C., D. Linch, et al. (1980). "Isolation of human haematopoietic progenitor cells using monoclonal antibodies." Nature **287**(5780): 332-333.

Bhardwaj, N. (2007). "Harnessing the immune system to treat cancer." J Clin Invest **117**(5): 1130-1136.

Biglarnia, A. R., B. Nilsson, et al. (2011). "Prompt reversal of a severe complement activation by eculizumab in a patient undergoing intentional ABO-incompatible pancreas and kidney transplantation." Transpl Int **24**(8): e61-66.

Bjorge, L., J. Hakulinen, et al. (2005). "Ascitic complement system in ovarian cancer." Br J Cancer **92**(5): 895-905.

Blom, A. M., B. O. Villoutreix, et al. (2004). "Complement inhibitor C4b-binding protein-friend or foe in the innate immune system?" Mol Immunol **40**(18): 1333-1346.

Blom, A. M., B. O. Villoutreix, et al. (2004). "Functions of human complement inhibitor C4b-binding protein in relation to its structure." Arch Immunol Ther Exp (Warsz) **52**(2): 83-95.

Bonavita, E., S. Gentile, et al. (2015). "PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer." Cell **160**(4): 700-714.

Boos, L., I. L. Campbell, et al. (2004). "Deletion of the complement anaphylatoxin C3a receptor attenuates, whereas ectopic expression of C3a in the brain exacerbates, experimental autoimmune encephalomyelitis." Journal of Immunology **173**(7): 4708-4714.

Boos, L., A. J. Szalai, et al. (2005). "C3a expressed in the central nervous system protects against LPS-induced shock." Neurosci Lett **387**(2): 68-71.

Borkowska, S., M. Suszynska, et al. (2014). "Novel evidence that crosstalk between the complement, coagulation and fibrinolysis proteolytic cascades is involved in mobilization of hematopoietic stem/progenitor cells (HSPCs)." Leukemia **28**(11): 2148-2154.

Bottazzi, B., A. Doni, et al. (2010). "An integrated view of humoral innate immunity: pentraxins as a paradigm." Annu Rev Immunol **28**: 157-183.

Bourgeois, C. and C. Tanchot (2003). "Mini-review CD4 T cells are required for CD8 T cell memory generation." European Journal of Immunology **33**(12): 3225-3231.

Boyer, M. W., P. J. Orchard, et al. (1995). "Dependency on intercellular adhesion molecule recognition and local interleukin-2 provision in generation of an in vivo CD8+ T-cell immune response to murine myeloid leukemia." Blood **85**(9): 2498-2506.

Brahmer, J. R., S. S. Tykodi, et al. (2012). "Safety and activity of anti-PD-L1 antibody in patients with advanced cancer." N Engl J Med **366**(26): 2455-2465.

Brasoveanu, L. I., M. Altomonte, et al. (1996). "Levels of cell membrane CD59 regulate the extent of complement-mediated lysis of human melanoma cells." Lab Invest **74**(1): 33-42.

Brown, D. A. and E. London (2000). "Structure and function of sphingolipid- and cholesterol-rich membrane rafts." J Biol Chem **275**(23): 17221-17224.

Brune, M., S. Castaigne, et al. (2006). "Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial." Blood **108**(1): 88-96.

Brune, M. and K. Hellstrand (1996). "Remission maintenance therapy with histamine and interleukin-2 in acute myelogenous leukaemia." Br J Haematol **92**(3): 620-626.

Brunet, J. F., F. Denizot, et al. (1987). "A new member of the immunoglobulin superfamily--CTLA-4." Nature **328**(6127): 267-270.

Bryceson, Y. T. and E. O. Long (2008). "Line of attack: NK cell specificity and integration of signals." Curr Opin Immunol **20**(3): 344-352.

Bryceson, Y. T., M. E. March, et al. (2006). "Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion." Blood **107**(1): 159-166.

Bu, X., Z. Zheng, et al. (2007). "Significance of C4d deposition in the follicular lymphoma and MALT lymphoma and their relationship with follicular dendritic cells." Pathol Res Pract **203**(3): 163-167.

Buessow, S. C., R. D. Paul, et al. (1984). "Influence of mammary tumor progression on phenotype and function of spleen and in situ lymphocytes in mice." J Natl Cancer Inst **73**(1): 249-255.

Bullinger, L., K. Dohner, et al. (2008). "An FLT3 gene-expression signature predicts clinical outcome in normal karyotype AML." Blood **111**(9): 4490-4495.

Burger, J. A., N. S. Velev, et al. (2010). "Failure is not fatal: long-term remission in refractory acute myeloid leukemia (AML) after graft failure of cord blood stem cells." Leukemia **24**(3): 666-668.

Burgi, B., T. Brunner, et al. (1994). "The degradation product of the C5a anaphylatoxin C5adesarg retains basophil-activating properties." Eur J Immunol **24**(7): 1583-1589.

Burnett, A. K., R. K. Hills, et al. (2011). "Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial." J Clin Oncol **29**(4): 369-377.

Buyse, M., P. Squifflet, et al. (2011). "Individual patient data meta-analysis of randomized trials evaluating IL-2 monotherapy as remission maintenance therapy in acute myeloid leukemia." Blood **117**(26): 7007-7013.

Calame, D. G., S. L. Mueller-Ortiz, et al. (2014). "The C5a anaphylatoxin receptor (C5aR1) protects against Listeria monocytogenes infection by inhibiting type 1 IFN expression." J Immunol **193**(10): 5099-5107.

Campbell, A. K., R. A. Daw, et al. (1981). "Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement." Biochem J **194**(2): 551-560.

Campbell, A. K. and B. P. Morgan (1985). "Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack." Nature **317**(6033): 164-166.

Carli, M., C. Bucolo, et al. (1979). "Fluctuation of serum complement levels in children with neuroblastoma." Cancer **43**(6): 2399-2404.

Carmi, Y., M. H. Spitzer, et al. (2015). "Allogeneic IgG combined with dendritic cell stimuli induce antitumour T-cell immunity." Nature **521**(7550): 99-104.

Carreras, J., A. Lopez-Guillermo, et al. (2006). "High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma." Blood **108**(9): 2957-2964.

Carroll, M. C. (2008). "Complement and humoral immunity." Vaccine **26 Suppl 8**: I28-33.

Caso, R., R. Silvera, et al. (2010). "Blood monocytes from mammary tumor-bearing mice: early targets of tumor-induced immune suppression?" Int J Oncol **37**(4): 891-900.

Chamuleau, M. E., G. J. Ossenkoppele, et al. (2011). "High TRAIL-R3 expression on leukemic blasts is associated with poor outcome and induces apoptosis-resistance which can be overcome by targeting TRAIL-R2." Leuk Res **35**(6): 741-749.

Chamuleau, M. E., A. A. van de Loosdrecht, et al. (2008). "High INDO (indoleamine 2,3-dioxygenase) mRNA level in blasts of acute myeloid leukemic patients predicts poor clinical outcome." Haematologica **93**(12): 1894-1898.

Chan, C. J., M. J. Smyth, et al. (2014). "Molecular mechanisms of natural killer cell activation in response to cellular stress." Cell Death Differ **21**(1): 5-14.

Chen, S., T. Caragine, et al. (2000). "CD59 expressed on a tumor cell surface modulates decay-accelerating factor expression and enhances tumor growth in a rat model of human neuroblastoma." Cancer Res **60**(11): 3013-3018.

Cheng, Z. Z., M. J. Corey, et al. (2005). "Complement factor H as a marker for detection of bladder cancer." Clin Chem **51**(5): 856-863.

Chien, S., Haq, S. U., Pawlus, M., Moon, R. T., Estey, E. H., Appelbaum, F. R., Othus, M., & Magnani, J. L. (2013). "Adhesion Of Acute Myeloid Leukemia Blasts To E-Selectin In The Vascular Niche Enhances Their Survival By Mechanisms Such As Wnt Activation." Blood **122**(21): 61.

Clark, R. A. and S. J. Klebanoff (1978). "Role of the classical and alternative complement pathways in chemotaxis and opsonization: studies of human serum deficient in C4." J Immunol **120**(4): 1102-1108.

Clynes, R. A., T. L. Towers, et al. (2000). "Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets." Nat Med **6**(4): 443-446.

Coca, S., J. Perez-Piqueras, et al. (1997). "The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma." Cancer **79**(12): 2320-2328.

Cole, D. S. and B. P. Morgan (2003). "Beyond lysis: how complement influences cell fate." Clin Sci (Lond) **104**(5): 455-466.

Coles, S. J., E. C. Wang, et al. (2011). "CD200 expression suppresses natural killer cell function and directly inhibits patient anti-tumor response in acute myeloid leukemia." Leukemia **25**(5): 792-799.

Cooley, S., D. J. Weisdorf, et al. (2014). "Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia." J Immunol **192**(10): 4592-4600.

Cooper, N. R. and H. J. Muller-Eberhard (1970). "The reaction mechanism of human C5 in immune hemolysis." J Exp Med **132**(4): 775-793.

Coral, S., E. Fonsatti, et al. (2000). "Overexpression of protectin (CD59) down-modulates the susceptibility of human melanoma cells to homologous complement." J Cell Physiol **185**(3): 317-323.

Cornacoff, J. B., L. A. Hebert, et al. (1984). "Factors influencing the binding of large immune complexes to the primate erythrocyte CR1 receptor." Clin Immunol Immunopathol **30**(2): 255-264.

Corrales, L., D. Ajona, et al. (2012). "Anaphylatoxin C5a creates a favorable microenvironment for lung cancer progression." Journal of Immunology **189**(9): 4674-4683.

Costello, R. T., S. Sivori, et al. (2002). "Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia." Blood **99**(10): 3661-3667.

Coulthard, L. G. and T. M. Woodruff (2015). "Is the complement activation product C3a a proinflammatory molecule? Re-evaluating the evidence and the myth." J Immunol **194**(8): 3542-3548.

Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature **420**(6917): 860-867.

Cragg, M. S., S. M. Morgan, et al. (2003). "Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts." Blood **101**(3): 1045-1052.

Cui, T., Y. Chen, et al. (2011). "Human complement factor H is a novel diagnostic marker for lung adenocarcinoma." Int J Oncol **39**(1): 161-168.

Curiel, T. J., G. Coukos, et al. (2004). "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival." Nat Med **10**(9): 942-949.

Curran, E., X. Chen, et al. (2016). "STING Pathway Activation Stimulates Potent Immunity against Acute Myeloid Leukemia." Cell Rep **15**(11): 2357-2366.

Curran, E., L. Corrales, et al. (2015). "Targeting the innate immune system as immunotherapy for acute myeloid leukemia." Front Oncol **5**: 83.

Curti, A., M. Aluigi, et al. (2007). "Acute myeloid leukemia cells constitutively express the immunoregulatory enzyme indoleamine 2,3-dioxygenase." Leukemia **21**(2): 353-355.

Curti, A., S. Trabanelli, et al. (2009). "The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: focus on hematology." Blood **113**(11): 2394-2401.

Da Costa, X. J., M. A. Brockman, et al. (1999). "Humoral response to herpes simplex virus is complement-dependent." Proc Natl Acad Sci U S A **96**(22): 12708-12712.

Daccache, A., T. Kizhakekuttu, et al. (2007). "Hematologic and cytogenetic spontaneous remission in acute monocytic leukemia (FAB M5b) with trisomy 8." J Clin Oncol **25**(3): 344-346.

Datta, J., J. H. Terhune, et al. (2014). "Optimizing dendritic cell-based approaches for cancer immunotherapy." Yale J Biol Med **87**(4): 491-518.

Davis, A. E., 3rd, P. Mejia, et al. (2008). "Biological activities of C1 inhibitor." Mol Immunol **45**(16): 4057-4063.

Deban, L., H. Jarva, et al. (2008). "Binding of the long pentraxin PTX3 to factor H: interacting domains and function in the regulation of complement activation." J Immunol **181**(12): 8433-8440.

Dechant, M., W. Weisner, et al. (2008). "Complement-dependent tumor cell lysis triggered by combinations of epidermal growth factor receptor antibodies." Cancer Res **68**(13): 4998-5003.

Del Poeta, G., A. Bruno, et al. (2008). "Deregulation of the mitochondrial apoptotic machinery and development of molecular targeted drugs in acute myeloid leukemia." Curr Cancer Drug Targets **8**(3): 207-222.

deLeeuw, R. J., S. E. Kost, et al. (2012). "The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature." Clin Cancer Res **18**(11): 3022-3029.

Dempsey, P. W., M. E. Allison, et al. (1996). "C3d of complement as a molecular adjuvant: bridging innate and acquired immunity." Science **271**(5247): 348-350.

Deng, L., H. Zhang, et al. (2010). "Accumulation of foxp3+ T regulatory cells in draining lymph nodes correlates with disease progression and immune suppression in colorectal cancer patients." Clin Cancer Res **16**(16): 4105-4112.

Devaud, C., P. K. Darcy, et al. (2014). "Foxp3 expression in T regulatory cells and other cell lineages." Cancer Immunol Immunother **63**(9): 869-876.

Di Gaetano, N., E. Cittera, et al. (2003). "Complement activation determines the therapeutic activity of rituximab in vivo." Journal of Immunology **171**(3): 1581-1587.

Diamond, M. S., M. Kinder, et al. (2011). "Type I interferon is selectively required by dendritic cells for immune rejection of tumors." J Exp Med **208**(10): 1989-2003.

Diermayr, S., H. Himmelreich, et al. (2008). "NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities." Blood **111**(3): 1428-1436.

Do, D. V., D. J. Pieramici, et al. (2014). "A phase ia dose-escalation study of the anti-factor D monoclonal antibody fragment FCFD4514S in patients with geographic atrophy." Retina **34**(2): 313-320.

Dohner, H., D. J. Weisdorf, et al. (2015). "Acute Myeloid Leukemia." N Engl J Med **373**(12): 1136-1152.

Dong, H., G. Zhu, et al. (1999). "B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion." Nat Med **5**(12): 1365-1369.

Downs-Canner, S., D. Magge, et al. (2015). "Complement Inhibition: A Novel Form of Immunotherapy for Colon Cancer." Ann Surg Oncol.

Drexler, H. G. (2001). The leukemia-lymphoma cell line. San Diego, Calif. ; London, Academic.

Droeser, R., I. Zlobec, et al. (2012). "Differential pattern and prognostic significance of CD4+, FOXP3+ and IL-17+ tumor infiltrating lymphocytes in ductal and lobular breast cancers." BMC Cancer **12**: 134.

Drouin, S. M., D. B. Corry, et al. (2002). "Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy." J Immunol **169**(10): 5926-5933.

Dunham, L. J. and H. L. Stewart (1953). "A survey of transplantable and transmissible animal tumors." J Natl Cancer Inst **13**(5): 1299-1377.

Dunkelberger, J. R. and W. C. Song (2010). "Complement and its role in innate and adaptive immune responses." Cell Res **20**(1): 34-50.

Dunn, G. P., A. T. Bruce, et al. (2002). "Cancer immunoediting: from immunosurveillance to tumor escape." Nature Immunology **3**(11): 991-998.

Dunn, G. P., A. T. Bruce, et al. (2005). "A critical function for type I interferons in cancer immunoediting." Nature Immunology **6**(7): 722-729.

Dunn, G. P., L. J. Old, et al. (2004). "The three Es of cancer immunoediting." Annu Rev Immunol **22**: 329-360.

Durrant, L. G., M. A. Chapman, et al. (2003). "Enhanced expression of the complement regulatory protein CD55 predicts a poor prognosis in colorectal cancer patients." Cancer Immunol Immunother **52**(10): 638-642.

Ehrengruber, M. U., T. Geiser, et al. (1994). "Activation of human neutrophils by C3a and C5A. Comparison of the effects on shape changes, chemotaxis, secretion, and respiratory burst." FEBS Lett **346**(2-3): 181-184.

el-Lati, S. G., C. A. Dahinden, et al. (1994). "Complement peptides C3a- and C5a-induced mediator release from dissociated human skin mast cells." J Invest Dermatol **102**(5): 803-806.

Elmaagacli, A. H., N. K. Steckel, et al. (2011). "Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients." Blood **118**(5): 1402-1412.

Elsner, J., M. Oppermann, et al. (1994). "C3a activates the respiratory burst in human polymorphonuclear neutrophilic leukocytes via pertussis toxin-sensitive G-proteins." Blood **83**(11): 3324-3331.

Ember, J. A., S. D. Sanderson, et al. (1992). "Biologic activity of synthetic analogues of C5a anaphylatoxin." J Immunol **148**(10): 3165-3173.

Ettinghausen, S. E., E. H. Lipford, 3rd, et al. (1985). "Recombinant interleukin 2 stimulates in vivo proliferation of adoptively transferred lymphokine-activated killer (LAK) cells." Journal of Immunology **135**(5): 3623-3635.

Falgarone, G. and G. Chiocchia (2009). "Chapter 8: Clusterin: A multifacet protein at the crossroad of inflammation and autoimmunity." Adv Cancer Res **104**: 139-170.

Farinha, P., A. Al-Tourah, et al. (2010). "The architectural pattern of FOXP3-positive T cells in follicular lymphoma is an independent predictor of survival and histologic transformation." Blood **115**(2): 289-295.

Fauriat, C., S. Just-Landi, et al. (2007). "Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction." Blood **109**(1): 323-330.

Fauriat, C., E. O. Long, et al. (2010). "Regulation of human NK-cell cytokine and chemokine production by target cell recognition." Blood **115**(11): 2167-2176.

Fearon, D. T. (1979). "Activation of the alternative complement pathway." CRC Crit Rev Immunol **1**(1): 1-32.

Fearon, D. T. (1979). "Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane." Proc Natl Acad Sci U S A **76**(11): 5867-5871.

Fearon, D. T. (1980). "Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte." J Exp Med **152**(1): 20-30.

Fearon, D. T. (1983). "The human C3b receptor." Springer Semin Immunopathol **6**(2-3): 159-172.

Fearon, D. T., I. Kaneko, et al. (1981). "Membrane distribution and adsorptive endocytosis by C3b receptors on human polymorphonuclear leukocytes." J Exp Med **153**(6): 1615-1628.

Fernandez Gonzalez, S., J. P. Jayasekera, et al. (2008). "Complement and natural antibody are required in the long-term memory response to influenza virus." Vaccine **26 Suppl 8**: I86-93.

Fife, B. T. and J. A. Bluestone (2008). "Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways." Immunol Rev **224**: 166-182.

Fischer, E., M. D. Appay, et al. (1986). "Characterization of the human glomerular C3 receptor as the C3b/C4b complement type one (CR1) receptor." Journal of Immunology **136**(4): 1373-1377.

Fischer, M. B., S. Goerg, et al. (1998). "Dependence of germinal center B cells on expression of CD21/CD35 for survival." Science **280**(5363): 582-585.

Fischer, W. H. and T. E. Hugli (1997). "Regulation of B cell functions by C3a and C3a(desArg): suppression of TNF-alpha, IL-6, and the polyclonal immune response." J Immunol **159**(9): 4279-4286.

Fishelson, Z., I. Hochman, et al. (2001). "Contribution of heat shock proteins to cell protection from complement-mediated lysis." International Immunology **13**(8): 983-991.

Flierman, R. and M. R. Daha (2007). "The clearance of apoptotic cells by complement." Immunobiology **212**(4-5): 363-370.

Fluiter, K., A. L. Opperhuizen, et al. (2014). "Inhibition of the membrane attack complex of the complement system reduces secondary neuroaxonal loss and promotes neurologic recovery after traumatic brain injury in mice." Journal of Immunology **192**(5): 2339-2348.

Foley, B., S. Cooley, et al. (2012). "Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen." Journal of Immunology **189**(10): 5082-5088.

Foley, B., S. Cooley, et al. (2012). "Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function." Blood **119**(11): 2665-2674.

Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nature Immunology **4**(4): 330-336.

Frade, R., M. C. Crevon, et al. (1985). "Enhancement of human B cell proliferation by an antibody to the C3d receptor, the gp 140 molecule." European Journal of Immunology **15**(1): 73-76.

Francis, K., B. M. Lewis, et al. (2003). "Complement C3a receptors in the pituitary gland: a novel pathway by which an innate immune molecule releases hormones involved in the control of inflammation." FASEB J **17**(15): 2266-2268.

Frey, D. M., R. A. Droeser, et al. (2010). "High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients." International Journal of Cancer **126**(11): 2635-2643.

Fuenmayor, J., K. Perez-Vazquez, et al. (2010). "Decreased survival of human breast cancer cells expressing HER2/neu on in vitro incubation with an anti-HER2/neu antibody fused to C5a or C5a desArg." Mol Cancer Ther **9**(8): 2175-2185.

Fuertes, M. B., A. K. Kacha, et al. (2011). "Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells." J Exp Med **208**(10): 2005-2016.

Fusakio, M. E., J. P. Mohammed, et al. (2011). "C5a regulates NKT and NK cell functions in sepsis." J Immunol **187**(11): 5805-5812.

Fust, G., Z. Miszlay, et al. (1987). "C1 and C4 abnormalities in chronic lymphocytic leukaemia and their significance." Immunol Lett **14**(3): 255-259.

Gabrilovich, D. I. and S. Nagaraj (2009). "Myeloid-derived suppressor cells as regulators of the immune system." Nat Rev Immunol **9**(3): 162-174.

Gajewski, T. F. (2007). "Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment." Clin Cancer Res **13**(18 Pt 1): 5256-5261.

Galon, J., A. Costes, et al. (2006). "Type, density, and location of immune cells within human colorectal tumors predict clinical outcome." Science **313**(5795): 1960-1964.

Gasiorowski, R. E., G. J. Clark, et al. (2014). "Antibody therapy for acute myeloid leukaemia." Br J Haematol **164**(4): 481-495.

Gasque, P., A. Thomas, et al. (1996). "Complement activation on human neuroblastoma cell lines in vitro: route of activation and expression of functional complement regulatory proteins." J Neuroimmunol **66**(1-2): 29-40.

Gay, L. J. and B. Felding-Habermann (2011). "Contribution of platelets to tumour metastasis." Nat Rev Cancer **11**(2): 123-134.

Gelderman, K. A., P. J. Kuppen, et al. (2002). "Enhancement of the complement activating capacity of 17-1A mAb to overcome the effect of membrane-bound complement regulatory proteins on colorectal carcinoma." European Journal of Immunology **32**(1): 128-135.

Gerard, C. and N. P. Gerard (1994). "C5A anaphylatoxin and its seven transmembrane-segment receptor." Annu Rev Immunol **12**: 775-808.

Ghannam, A., M. Pernollet, et al. (2008). "Human C3 deficiency associated with impairments in dendritic cell differentiation, memory B cells, and regulatory T cells." Journal of Immunology **181**(7): 5158-5166.

Gibbins, J. D., L. R. Ancelet, et al. (2014). "An autologous leukemia cell vaccine prevents murine acute leukemia relapse after cytarabine treatment." Blood **124**(19): 2953-2963.

Gminski, J., J. Mykala-Ciesla, et al. (1992). "Immunoglobulins and complement components levels in patients with lung cancer." Rom J Intern Med **30**(1): 39-44.

Goldie, H., C. H. Butler, et al. (1953). "Growth characteristics of free C1498 granulocytic leukemia tumor cells in the peritoneal fluid and the blood of C57 mice." Cancer Research **13**(2): 125-129.

Gooley, T. A., J. W. Chien, et al. (2010). "Reduced mortality after allogeneic hematopoietic-cell transplantation." N Engl J Med **363**(22): 2091-2101.

Goswami, D., K. Gowrishankar, et al. (2008). "Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity." Cell **135**(6): 1085-1097.

Greiner, J., L. Bullinger, et al. (2008). "Leukemia-associated antigens are critical for the proliferation of acute myeloid leukemia cells." Clin Cancer Res **14**(22): 7161-7166.

Grimwade, D., J. V. Jovanovic, et al. (2014). "Can we say farewell to monitoring minimal residual disease in acute promyelocytic leukaemia?" Best Pract Res Clin Haematol **27**(1): 53-61.

Grimwade, D., J. V. Jovanovic, et al. (2009). "Prospective minimal residual disease monitoring to predict relapse of acute promyelocytic leukemia and to direct pre-emptive arsenic trioxide therapy." J Clin Oncol **27**(22): 3650-3658.

Grivennikov, S. I., F. R. Greten, et al. (2010). "Immunity, inflammation, and cancer." Cell **140**(6): 883-899.

Groh, V., J. Wu, et al. (2002). "Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation." Nature **419**(6908): 734-738.

Grossman, T. R., L. A. Hettrick, et al. (2016). "Inhibition of the alternative complement pathway by antisense oligonucleotides targeting complement factor B improves lupus nephritis in mice." Immunobiology **221**(6): 701-708.

Grosso, D. A., R. C. Hess, et al. (2015). "Immunotherapy in acute myeloid leukemia." Cancer **121**(16): 2689-2704.

Grulich, A. E., M. T. van Leeuwen, et al. (2007). "Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis." Lancet **370**(9581): 59-67.

Guglietta, S., A. Chiavelli, et al. (2016). "Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis." Nat Commun **7**: 11037.

Guidi, L., R. Baroni, et al. (1988). "Immune complexes in solid tumours precipitable by 3.5% polyethylene glycol: analysis of some nonspecific components." Diagn Clin Immunol **5**(6): 284-288.

Gunn, L., C. Ding, et al. (2012). "Opposing roles for complement component C5a in tumor progression and the tumor microenvironment." Journal of Immunology **189**(6): 2985-2994.

Gupta, V., M. S. Tallman, et al. (2011). "Allogeneic hematopoietic cell transplantation for adults with acute myeloid leukemia: myths, controversies, and unknowns." Blood **117**(8): 2307-2318.

Gyapay, G., B. Schmidt, et al. (1991). "Effect of conditioned media of acute myeloid leukemia blast cells on complement synthesis by cultured human cells of monocyte and hepatocyte origin." Complement Inflamm **8**(5-6): 370-377.

Haas, K. M., M. Hasegawa, et al. (2002). "Complement receptors CD21/35 link innate and protective immunity during Streptococcus pneumoniae infection by regulating IgG3 antibody responses." Immunity **17**(6): 713-723.

Haas, P. J. and J. van Strijp (2007). "Anaphylatoxins: their role in bacterial infection and inflammation." Immunol Res **37**(3): 161-175.

Hajishengallis, G. and J. D. Lambris (2010). "Crosstalk pathways between Toll-like receptors and the complement system." Trends Immunol **31**(4): 154-163.

Hallett, M. B., J. P. Luzio, et al. (1981). "Stimulation of Ca2+-dependent chemiluminescence in rat polymorphonuclear leucocytes by polystyrene beads and the non-lytic action of complement." Immunology **44**(3): 569-576.

Hamad, O. A., I. Mitroulis, et al. (2015). "Contact activation of C3 enables tethering between activated platelets and polymorphonuclear leukocytes via CD11b/CD18." Thromb Haemost **114**(6): 1207-1217.

Hamanishi, J., M. Mandai, et al. (2007). "Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer." Proc Natl Acad Sci U S A **104**(9): 3360-3365.

Hamuro, J., U. Hadding, et al. (1978). "Fragments Ba and Bb derived from guinea pig factor B of the properdin system: purification, characterization, and biologic activities." J Immunol **120**(2): 438-444.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hancock, J. F. (2006). "Lipid rafts: contentious only from simplistic standpoints." Nat Rev Mol Cell Biol **7**(6): 456-462.

Hannani, D., C. Locher, et al. (2014). "Contribution of humoral immune responses to the antitumor effects mediated by anthracyclines." Cell Death Differ **21**(1): 50-58.

Harder, T., P. Scheiffele, et al. (1998). "Lipid domain structure of the plasma membrane revealed by patching of membrane components." J Cell Biol **141**(4): 929-942.

Heine, H., E. T. Rietschel, et al. (2001). "The biology of endotoxin." Mol Biotechnol **19**(3): 279-296.

Herberman, R. B., M. E. Nunn, et al. (1975). "Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells." Int J Cancer **16**(2): 230-239.

Hernandez-Caselles, T., M. Martinez-Esparza, et al. (2006). "A study of CD33 (SIGLEC-3) antigen expression and function on activated human T and NK cells: two isoforms of CD33 are generated by alternative splicing." J Leukoc Biol **79**(1): 46-58.

Heyman, B., E. J. Wiersma, et al. (1990). "In vivo inhibition of the antibody response by a complement receptor-specific monoclonal antibody." J Exp Med **172**(2): 665-668.

Hill, A., R. P. Rother, et al. (2010). "Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization." Haematologica **95**(4): 567-573.

Hillmen, P., N. S. Young, et al. (2006). "The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria." N Engl J Med **355**(12): 1233-1243.

Hofman, P., B. L. Hsi, et al. (1994). "High expression of the antigen recognized by the monoclonal antibody GB24 on human breast carcinomas: a preventive mechanism of malignant tumor cells against complement attack?" Breast Cancer Res Treat **32**(2): 213-219.

Hogg, N. (1992). "Roll, roll, roll your leucocyte gently down the vein." Immunol Today **13**(4): 113-115.

Hollmann, T. J., S. L. Mueller-Ortiz, et al. (2008). "Disruption of the C5a receptor gene increases resistance to acute Gram-negative bacteremia and endotoxic shock: opposing roles of C3a and C5a." Molecular Immunology **45**(7): 1907-1915.

Holmberg, M. T., A. M. Blom, et al. (2001). "Regulation of complement classical pathway by association of C4b-binding protein to the surfaces of SK-OV-3 and Caov-3 ovarian adenocarcinoma cells." Journal of Immunology **167**(2): 935-939.

Hong, F., R. D. Hansen, et al. (2003). "Beta-glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells." Cancer Res **63**(24): 9023-9031.

Hori, S., T. Nomura, et al. (2003). "Control of regulatory T cell development by the transcription factor Foxp3." Science **299**(5609): 1057-1061.

Hossain, D. M., C. Dos Santos, et al. (2014). "Leukemia cell-targeted STAT3 silencing and TLR9 triggering generate systemic antitumor immunity." Blood **123**(1): 15-25.

Hu, X., V. M. Holers, et al. (2013). "Therapeutic inhibition of the alternative complement pathway attenuates chronic EAE." Molecular Immunology **54**(3-4): 302-308.

Hu, Y., M. J. Turner, et al. (2009). "Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model." Immunology **128**(2): 260-270.

Huber-Lang, M., J. V. Sarma, et al. (2005). "Changes in the novel orphan, C5a receptor (C5L2), during experimental sepsis and sepsis in humans." Journal of Immunology **174**(2): 1104-1110.

Huber-Lang, M., J. V. Sarma, et al. (2006). "Generation of C5a in the absence of C3: a new complement activation pathway." Nat Med **12**(6): 682-687.

Hugli, T. E., M. S. Kawahara, et al. (1983). "The active site of human C4a anaphylatoxin." Molecular Immunology **20**(6): 637-645.

Iijima, N., K. Miyamura, et al. (1997). "Functional expression of Fas (CD95) in acute myeloid leukemia cells in the context of CD34 and CD38 expression: possible correlation with sensitivity to chemotherapy." Blood **90**(12): 4901-4909.

Imai, M., C. Landen, et al. (2005). "Complement-mediated mechanisms in anti-GD2 monoclonal antibody therapy of murine metastatic cancer." Cancer Research **65**(22): 10562-10568.

Imamura, T., M. Yamamoto-Ibusuki, et al. (2015). "Influence of the C5a-C5a receptor system on breast cancer progression and patient prognosis." Breast Cancer.

Jalili, A., N. Shirvaikar, et al. (2010). "Fifth complement cascade protein (C5) cleavage fragments disrupt the SDF-1/CXCR4 axis: further evidence that innate immunity orchestrates the mobilization of hematopoietic stem/progenitor cells." Exp Hematol **38**(4): 321-332.

Janelle, V., M. P. Langlois, et al. (2014). "Transient complement inhibition promotes a tumor-specific immune response through the implication of natural killer cells." Cancer Immunol Res **2**(3): 200-206.

Janssen, E. M., E. E. Lemmens, et al. (2003). "CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes." Nature **421**(6925): 852-856.

Jarvis, G. A., J. Li, et al. (1997). "Expression and function of the complement membrane attack complex inhibitor protectin (CD59) in human prostate cancer." Int J Cancer **71**(6): 1049-1055.

Jenkins, M. K., D. M. Pardoll, et al. (1987). "T-cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state." Immunol Rev **95**: 113-135.

Jenne, D., A. Hille, et al. (1989). "Sulfation of two tyrosine-residues in human complement S-protein (vitronectin)." Eur J Biochem **185**(2): 391-395.

Jenne, D. E. and J. Tschopp (1989). "Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid." Proc Natl Acad Sci U S A **86**(18): 7123-7127.

Jozsi, M. and P. F. Zipfel (2008). "Factor H family proteins and human diseases." Trends Immunol **29**(8): 380-387.

Junnikkala, S., J. Hakulinen, et al. (2002). "Secretion of soluble complement inhibitors factor H and factor H-like protein (FHL-1) by ovarian tumour cells." Br J Cancer **87**(10): 1119-1127.

Junnikkala, S., T. S. Jokiranta, et al. (2000). "Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1." Journal of Immunology **164**(11): 6075-6081.

Jurianz, K., S. Ziegler, et al. (2001). "K562 erythroleukemic cells are equipped with multiple mechanisms of resistance to lysis by complement." International Journal of Cancer **93**(6): 848-854.

Kaech, S. M., S. Hemby, et al. (2002). "Molecular and functional profiling of memory CD8 T cell differentiation." Cell **111**(6): 837-851.

Kalant, D., S. A. Cain, et al. (2003). "The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg77/acylation-stimulating protein." J Biol Chem **278**(13): 11123-11129.

Kalli, K. R. and D. T. Fearon (1994). "Binding of C3b and C4b by the CR1-like site in murine CR1." Journal of Immunology **152**(6): 2899-2903.

Kaufman, D. S., R. A. Schoon, et al. (1995). "Inhibition of selective signaling events in natural killer cells recognizing major histocompatibility complex class I." Proc Natl Acad Sci U S A **92**(14): 6484-6488.

Kaya, Z., M. Afanasyeva, et al. (2001). "Contribution of the innate immune system to autoimmune myocarditis: a role for complement." Nat Immunol **2**(8): 739-745.

Kayser, S., R. F. Schlenk, et al. (2015). "Minimal residual disease-directed therapy in acute myeloid leukemia." Blood **125**(15): 2331-2335.

Keilholz, U., A. Letsch, et al. (2009). "A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS." Blood **113**(26): 6541-6548.

Kemper, C., A. C. Chan, et al. (2003). "Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype." Nature **421**(6921): 388-392.

Kempshall, E., S. Thebault, et al. (2012). "Complement-induced protection: an explanation for the limitations of cell-based tumour immunotherapies." Immunol Cell Biol **90**(9): 869-871.

Kennedy, A. D., M. D. Solga, et al. (2003). "An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20+ cells by rituximab." Blood **101**(3): 1071-1079.

Khong, H. T. and N. P. Restifo (2002). "Natural selection of tumor variants in the generation of "tumor escape" phenotypes." Nature Immunology **3**(11): 999-1005.

Kiessling, R., E. Klein, et al. (1975). ""Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell." Eur J Immunol **5**(2): 117-121.

Kim, D. Y., C. B. Martin, et al. (2005). "Expression of complement protein C5a in a murine mammary cancer model: tumor regression by interference with the cell cycle." Cancer Immunol Immunother **54**(10): 1026-1037.

Kim, J. H., S. J. Oh, et al. (2014). "IFN-gamma-producing NKT cells exacerbate sepsis by enhancing C5a generation via IL-10-mediated inhibition of CD55 expression on neutrophils." Eur J Immunol **44**(7): 2025-2035.

Kim, S., K. Iizuka, et al. (2000). "In vivo natural killer cell activities revealed by natural killer cell-deficient mice." Proc Natl Acad Sci U S A **97**(6): 2731-2736.

Kinders, R., T. Jones, et al. (1998). "Complement factor H or a related protein is a marker for transitional cell cancer of the bladder." Clin Cancer Res **4**(10): 2511-2520.

Kitano, E. and H. Kitamura (1993). "Synthesis of the third component of complement (C3) by human gastric cancer-derived cell lines." Clinical and Experimental Immunology **94**(2): 273-278.

Klepfish, A., L. Gilles, et al. (2009). "Enhancing the action of rituximab in chronic lymphocytic leukemia by adding fresh frozen plasma: complement/rituximab interactions & clinical results in refractory CLL." Ann N Y Acad Sci **1173**: 865-873.

Koeffler, H. P. and D. W. Golde (1980). "Human myeloid leukemia cell lines: a review." Blood **56**(3): 344-350.

Kohl, J. (2006). "Self, non-self, and danger: a complementary view." Adv Exp Med Biol **586**: 71-94.

Kohl, J., R. Baelder, et al. (2006). "A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma." J Clin Invest **116**(3): 783-796.

Kojouharova, M., K. Reid, et al. (2010). "New insights into the molecular mechanisms of classical complement activation." Molecular Immunology **47**(13): 2154-2160.

Kopf, M., B. Abel, et al. (2002). "Complement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection." Nat Med **8**(4): 373-378.

Koretz, K., S. Bruderlein, et al. (1993). "Expression of CD59, a complement regulator protein and a second ligand of the CD2 molecule, and CD46 in normal and neoplastic colorectal epithelium." Br J Cancer **68**(5): 926-931.

Koski, C. L., L. E. Ramm, et al. (1983). "Cytolysis of nucleated cells by complement: cell death displays multi-hit characteristics." Proc Natl Acad Sci U S A **80**(12): 3816-3820.

Kozono, H., T. Kinoshita, et al. (1990). "Localization of the covalent C3b-binding site on C4b within the complement classical pathway C5 convertase, C4b2a3b." J Biol Chem **265**(24): 14444-14449.

Kraus, S. and Z. Fishelson (2000). "Cell desensitization by sublytic C5b-9 complexes and calcium ionophores depends on activation of protein kinase C." European Journal of Immunology **30**(5): 1272-1280.

Kraus, S., R. Seger, et al. (2001). "Involvement of the ERK mitogen-activated protein kinase in cell resistance to complement-mediated lysis." Clinical and Experimental Immunology **123**(3): 366-374.

Kremer, K. N., A. Dudakovic, et al. (2014). "Osteoblasts protect AML cells from SDF-1-induced apoptosis." J Cell Biochem **115**(6): 1128-1137.

Kretzschmar, T., A. Jeromin, et al. (1993). "Chronic myelogenous leukemia-derived basophilic granulocytes express a functional active receptor for the anaphylatoxin C3a." Eur J Immunol **23**(2): 558-561.

Krummel, M. F. and J. P. Allison (1995). "CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation." J Exp Med **182**(2): 459-465.

Krych-Goldberg, M. and J. P. Atkinson (2001). "Structure-function relationships of complement receptor type 1." Immunol Rev **180**: 112-122.

Kumar, V., S. R. Ali, et al. (2006). "Cell-derived anaphylatoxins as key mediators of antibody-dependent type II autoimmunity in mice." J Clin Invest **116**(2): 512-520.

Kung Sutherland, M. S., R. B. Walter, et al. (2013). "SGN-CD33A: a novel CD33-targeting antibody-drug conjugate using a pyrrolobenzodiazepine dimer is active in models of drug-resistant AML." Blood **122**(8): 1455-1463.

Kusmartsev, S., S. Nagaraj, et al. (2005). "Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells." Journal of Immunology **175**(7): 4583-4592.

Ladoire, S., F. Martin, et al. (2011). "Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer." Cancer Immunol Immunother **60**(7): 909-918.

Lalli, P. N., M. G. Strainic, et al. (2008). "Locally produced C5a binds to T cell-expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis." Blood **112**(5): 1759-1766.

Lambris, J. D. (1988). "The multifunctional role of C3, the third component of complement." Immunol Today **9**(12): 387-393.

Lambris, J. D. and H. J. Muller-Eberhard (1986). "The multifunctional role of C3: structural analysis of its interactions with physiological ligands." Molecular Immunology **23**(11): 1237-1242.

Langer, H. F., K. J. Chung, et al. (2010). "Complement-mediated inhibition of neovascularization reveals a point of convergence between innate immunity and angiogenesis." Blood **116**(22): 4395-4403.

Lanier, L. L., J. J. Ruitenberg, et al. (1988). "Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes." Journal of Immunology **141**(10): 3478-3485.

Larkin, J., V. Chiarion-Sileni, et al. (2015). "Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma." N Engl J Med **373**(1): 23-34.

Laudisi, F., R. Spreafico, et al. (2013). "Cutting edge: the NLRP3 inflammasome links complement-mediated inflammation and IL-1beta release." J Immunol **191**(3): 1006-1010.

Le Dieu, R., D. C. Taussig, et al. (2009). "Peripheral blood T cells in acute myeloid leukemia (AML) patients at diagnosis have abnormal phenotype and genotype and form defective immune synapses with AML blasts." Blood **114**(18): 3909-3916.

Legoedec, J., P. Gasque, et al. (1995). "Expression of the complement alternative pathway by human myoblasts in vitro: biosynthesis of C3, factor B, factor H and factor I." European Journal of Immunology **25**(12): 3460-3466.

Li, B., D. J. Allendorf, et al. (2007). "Combined yeast {beta}-glucan and antitumor monoclonal antibody therapy requires C5a-mediated neutrophil chemotaxis via regulation of decay-accelerating factor CD55." Cancer Research **67**(15): 7421-7430.

Li, K., K. J. Anderson, et al. (2008). "Cyclic AMP plays a critical role in C3a-receptor-mediated regulation of dendritic cells in antigen uptake and T-cell stimulation." Blood **112**(13): 5084-5094.

Li, R., L. G. Coulthard, et al. (2013). "C5L2: a controversial receptor of complement anaphylatoxin, C5a." FASEB J **27**(3): 855-864.

Liang, T., N. Wang, et al. (2010). "Identification of complement C3f-desArg and its derivative for acute leukemia diagnosis and minimal residual disease assessment." Proteomics **10**(1): 90-98.

Lim, S. H., C. P. Worman, et al. (1991). "Production of tumour-derived suppressor factor in patients with acute myeloid leukaemia." Leuk Res **15**(4): 263-268.

Lin, W. W. and M. Karin (2007). "A cytokine-mediated link between innate immunity, inflammation, and cancer." J Clin Invest **117**(5): 1175-1183.

Lingwood, D. and K. Simons (2010). "Lipid rafts as a membrane-organizing principle." Science **327**(5961): 46-50.

Linsley, P. S., W. Brady, et al. (1991). "CTLA-4 is a second receptor for the B cell activation antigen B7." J Exp Med **174**(3): 561-569.

Liszewski, M. K., M. Kolev, et al. (2013). "Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation." Immunity **39**(6): 1143-1157.

Liszewski, M. K., T. W. Post, et al. (1991). "Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster." Annu Rev Immunol **9**: 431-455.

Loberg, R. D., K. J. Wojno, et al. (2005). "Analysis of membrane-bound complement regulatory proteins in prostate cancer." Urology **66**(6): 1321-1326.

Loveland, B. E. and J. Cebon (2008). "Cancer exploiting complement: a clue or an exception?" Nature Immunology **9**(11): 1205-1206.

Lublin, D. M. and J. P. Atkinson (1989). "Decay-accelerating factor: biochemistry, molecular biology, and function." Annu Rev Immunol **7**: 35-58.

Lucas, S. D., A. Karlsson-Parra, et al. (1996). "Tumor-specific deposition of immunoglobulin G and complement in papillary thyroid carcinoma." Hum Pathol **27**(12): 1329-1335.

Lupu, F., R. S. Keshari, et al. (2014). "Crosstalk between the coagulation and complement systems in sepsis." Thromb Res **133 Suppl 1**: S28-31.

Madjd, Z., L. G. Durrant, et al. (2004). "Loss of CD55 is associated with aggressive breast tumors." Clin Cancer Res **10**(8): 2797-2803.

Madjd, Z., L. G. Durrant, et al. (2005). "Do poor-prognosis breast tumours express membrane cofactor proteins (CD46)?" Cancer Immunol Immunother **54**(2): 149-156.

Mak, I. W., N. Evaniew, et al. (2014). "Lost in translation: animal models and clinical trials in cancer treatment." Am J Transl Res **6**(2): 114-118.

Mandell, G. L., J. E. Bennett, et al. (2000). Mandell, Douglas and Bennett's principles and practice of infectious diseases. Philadelphia ; London, Churchill Livingstone.

Maness, P. F. and A. Orengo (1977). "Serum complement levels in patients with digestive tract carcinomas and other neoplastic diseases." Oncology **34**(2): 87-89.

Mantovani, A., P. Romero, et al. (2008). "Tumour immunity: effector response to tumour and role of the microenvironment." Lancet **371**(9614): 771-783.

Marchbank, K. J., C. W. van den Berg, et al. (1997). "Mechanisms of complement resistance induced by non-lethal complement attack and by growth arrest." Immunology **90**(4): 647-653.

Mardis, E. R., L. Ding, et al. (2009). "Recurring mutations found by sequencing an acute myeloid leukemia genome." N Engl J Med **361**(11): 1058-1066.

Markiewski, M. M., R. A. DeAngelis, et al. (2008). "Modulation of the antitumor immune response by complement." Nature Immunology **9**(11): 1225-1235.

Markiewski, M. M., R. A. DeAngelis, et al. (2009). "The regulation of liver cell survival by complement." Journal of Immunology **182**(9): 5412-5418.

Markiewski, M. M., B. Nilsson, et al. (2007). "Complement and coagulation: strangers or partners in crime?" Trends Immunol **28**(4): 184-192.

Marsh, J. E., C. K. Farmer, et al. (2001). "The allogeneic T and B cell response is strongly dependent on complement components C3 and C4." Transplantation **72**(7): 1310-1318.

Martin, P. J., J. A. Ledbetter, et al. (1986). "A 44 kilodalton cell surface homodimer regulates interleukin 2 production by activated human T lymphocytes." Journal of Immunology **136**(9): 3282-3287.

Martin, U., D. Bock, et al. (1997). "The human C3a receptor is expressed on neutrophils and monocytes, but not on B or T lymphocytes." J Exp Med **186**(2): 199-207.

Martner, A., A. Rydstrom, et al. (2016). "Role of natural killer cell subsets and natural cytotoxicity receptors for the outcome of immunotherapy in acute myeloid leukemia." Oncoimmunology **5**(1): e1041701.

Mastellos, D. C., D. Yancopoulou, et al. (2015). "Compstatin: a C3-targeted complement inhibitor reaching its prime for bedside intervention." Eur J Clin Invest **45**(4): 423-440.

Matheoud, D., L. Perie, et al. (2010). "Cross-presentation by dendritic cells from live cells induces protective immune responses in vivo." Blood **115**(22): 4412-4420.

Matsutani, M., T. Suzuki, et al. (1984). "Cellular immunity and complement levels in hosts with brain tumours." Neurosurg Rev **7**(1): 29-35.

McClune, B. L., D. J. Weisdorf, et al. (2010). "Effect of age on outcome of reduced-intensity hematopoietic cell transplantation for older patients with acute myeloid leukemia in first complete remission or with myelodysplastic syndrome." J Clin Oncol **28**(11): 1878-1887.

Medof, M. E., E. I. Walter, et al. (1987). "Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids." J Exp Med **165**(3): 848-864.

Melero, I., G. Gaudernack, et al. (2014). "Therapeutic vaccines for cancer: an overview of clinical trials." Nat Rev Clin Oncol **11**(9): 509-524.

Meri, S., B. P. Morgan, et al. (1990). "Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers." Immunology **71**(1): 1-9.

Metzeler, K. H., M. Hummel, et al. (2008). "An 86-probe-set gene-expression signature predicts survival in cytogenetically normal acute myeloid leukemia." Blood **112**(10): 4193-4201.

Min, X., C. Liu, et al. (2014). "Expression and regulation of complement receptors by human natural killer cells." Immunobiology **219**(9): 671-679.

Min, Y. J., J. H. Lee, et al. (2004). "Prognostic significance of Fas (CD95) and TRAIL receptors (DR4/DR5) expression in acute myelogenous leukemia." Leuk Res **28**(4): 359-365.

Minard-Colin, V., Y. Xiu, et al. (2008). "Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage FcgammaRI, FcgammaRIII, and FcgammaRIV." Blood **112**(4): 1205-1213.

Minh, D. Q., E. Czink, et al. (1983). "Serial complement measurements in patients with leukaemia." Clin Lab Haematol **5**(1): 23-34.

Mitterbauer, M., M. Fritzer-Szekeres, et al. (1996). "Spontaneous remission of acute myeloid leukemia after infection and blood transfusion associated with hypergammaglobulinaemia." Ann Hematol **73**(4): 189-193.

Monsurro, V., E. Wang, et al. (2004). "Quiescent phenotype of tumor-specific CD8+ T cells following immunization." Blood **104**(7): 1970-1978.

Morel, P. A., L. K. Ernst, et al. (1999). "Functional CD32 molecules on human NK cells." Leuk Lymphoma **35**(1-2): 47-56.

Morgan, B. P. (1990). Complement : clinical aspects and relevance to disease, Academic Press.

Morgan, B. P. (1999). "Regulation of the complement membrane attack pathway." Crit Rev Immunol **19**(3): 173-198.

Morgan, B. P. and A. K. Campbell (1985). "The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium." Biochem J **231**(1): 205-208.

Morgan, B. P., J. Chamberlain-Banoub, et al. (2006). "The membrane attack pathway of complement drives pathology in passively induced experimental autoimmune myasthenia gravis in mice." Clin Exp Immunol **146**(2): 294-302.

Morgan, B. P., J. R. Dankert, et al. (1987). "Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis." Journal of Immunology **138**(1): 246-253.

Morgan, B. P. and P. Gasque (1997). "Extrahepatic complement biosynthesis: where, when and why?" Clinical and Experimental Immunology **107**(1): 1-7.

Morgan, B. P. and C. L. Harris (2003). "Complement therapeutics; history and current progress." Molecular Immunology **40**(2-4): 159-170.

Morgan, B. P. and S. Meri (1994). "Membrane-Proteins That Protect against Complement Lysis." Springer Seminars in Immunopathology **15**(4): 369-396.

Morgan, B. P. and M. J. Walport (1991). "Complement deficiency and disease." Immunol Today **12**(9): 301-306.

Morris, E., D. Hart, et al. (2006). "Generation of tumor-specific T-cell therapies." Blood Rev **20**(2): 61-69.

Moskovich, O. and Z. Fishelson (2007). "Live cell imaging of outward and inward vesiculation induced by the complement c5b-9 complex." J Biol Chem **282**(41): 29977-29986.

Mougiakakos, D., A. Choudhury, et al. (2010). "Regulatory T cells in cancer." Adv Cancer Res **107**: 57-117.

Muller, C. I., M. Trepel, et al. (2004). "Hematologic and molecular spontaneous remission following sepsis in acute monoblastic leukemia with translocation (9;11): a case report and review of the literature." Eur J Haematol **73**(1): 62-66.

Murakami, Y., T. Imamichi, et al. (1993). "Characterization of C3a anaphylatoxin receptor on guinea-pig macrophages." Immunology **79**(4): 633-638.

Mural, R. J., M. D. Adams, et al. (2002). "A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome." Science **296**(5573): 1661-1671.

Murdoch, C., M. Muthana, et al. (2008). "The role of myeloid cells in the promotion of tumour angiogenesis." Nat Rev Cancer **8**(8): 618-631.

Murphy, K., P. Travers, et al. Janeway's immunobiology. New York, Garland Science ; London : Taylor & Francis [distributor].

Mussai, F., C. De Santo, et al. (2013). "Acute myeloid leukemia creates an arginase-dependent immunosuppressive microenvironment." Blood **122**(5): 749-758.

Myones, B. L., J. G. Dalzell, et al. (1988). "Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3." J Clin Invest **82**(2): 640-651.

Nabizadeh, J. A., H. D. Manthey, et al. (2016). "The Complement C3a Receptor Contributes to Melanoma Tumorigenesis by Inhibiting Neutrophil and CD4+ T Cell Responses." J Immunol **196**(11): 4783-4792.

Nagasawa, S. and R. M. Stroud (1977). "Cleavage of C2 by C1s into the antigenically distinct fragments C2a and C2b: demonstration of binding of C2b to C4b." Proc Natl Acad Sci U S A **74**(7): 2998-3001.

Nakayama, Y., S. I. Kim, et al. (2009). "C3 promotes expansion of CD8+ and CD4+ T cells in a Listeria monocytogenes infection." J Immunol **183**(5): 2921-2931.

Nataf, S., N. Davoust, et al. (1999). "Human T cells express the C5a receptor and are chemoattracted to C5a." J Immunol **162**(7): 4018-4023.

Nauts, H. C. and J. R. McLaren (1990). "Coley toxins--the first century." Adv Exp Med Biol **267**: 483-500.

Nicol, P. A. and P. J. Lachmann (1973). "The alternate pathway of complement activation. The role of C3 and its inactivator (KAF)." Immunology **24**(2): 259-275.

Niculescu, F., T. Badea, et al. (1999). "Sublytic C5b-9 induces proliferation of human aortic smooth muscle cells: role of mitogen activated protein kinase and phosphatidylinositol 3-kinase." Atherosclerosis **142**(1): 47-56.

Niculescu, F., H. G. Rus, et al. (1992). "Persistent complement activation on tumor cells in breast cancer." Am J Pathol **140**(5): 1039-1043.

Niehans, G. A., D. L. Cherwitz, et al. (1996). "Human carcinomas variably express the complement inhibitory proteins CD46 (membrane cofactor protein), CD55 (decay-accelerating factor), and CD59 (protectin)." Am J Pathol **149**(1): 129-142.

Nishioka, K., K. Kawamura, et al. (1976). "The complement system in tumor immunity: significance of elevated levels of complement in tumor bearing hosts." Ann N Y Acad Sci **276**: 303-315.

Nomi, T., M. Sho, et al. (2007). "Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer." Clin Cancer Res **13**(7): 2151-2157.

Nunez-Cruz, S., P. A. Gimotty, et al. (2012). "Genetic and pharmacologic inhibition of complement impairs endothelial cell function and ablates ovarian cancer neovascularization." Neoplasia **14**(11): 994-1004.

Ohanian, S. H. and S. I. Schlager (1981). "Humoral immune killing of nucleated cells: mechanisms of complement-mediated attack and target cell defense." Crit Rev Immunol **1**(3): 165-209.

Oka, Y., A. Tsuboi, et al. (2004). "Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression." Proc Natl Acad Sci U S A **101**(38): 13885-13890.

Okinaga, S., D. Slattery, et al. (2003). "C5L2, a nonsignaling C5A binding protein." Biochemistry **42**(31): 9406-9415.

Okroj, M., Y. F. Hsu, et al. (2008). "Non-small cell lung cancer cells produce a functional set of complement factor I and its soluble cofactors." Mol Immunol **45**(1): 169-179.

Ollert, M. W., K. David, et al. (1995). "Classical complement pathway activation on nucleated cells. Role of factor H in the control of deposited C3b." J Immunol **155**(10): 4955-4962.

Ollert, M. W., J. V. Kadlec, et al. (1994). "Antibody-mediated complement activation on nucleated cells. A quantitative analysis of the individual reaction steps." Journal of Immunology **153**(5): 2213-2221.

Ondondo, B., E. Jones, et al. (2013). "Home sweet home: the tumor microenvironment as a haven for regulatory T cells." Front Immunol **4**: 197.

Ottonello, L., A. Corcione, et al. (1999). "rC5a directs the in vitro migration of human memory and naive tonsillar B lymphocytes: implications for B cell trafficking in secondary lymphoid tissues." J Immunol **162**(11): 6510-6517.

Papadimitriou, J. C., D. F. Carney, et al. (1991). "Inhibitors of membrane lipid metabolism enhance complement-mediated nucleated cell killing through distinct mechanisms." Molecular Immunology **28**(8): 803-809.

Papamichail, M., C. Gutierrez, et al. (1975). "Complement dependence of localisation of aggregated IgG in germinal centres." Scand J Immunol **4**(4): 343-347.

Pardoll, D. M. (2012). "The blockade of immune checkpoints in cancer immunotherapy." Nat Rev Cancer **12**(4): 252-264.

Pearson, G., F. Robinson, et al. (2001). "Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions." Endocr Rev **22**(2): 153-183.

Peled, A., O. Kollet, et al. (2000). "The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice." Blood **95**(11): 3289-3296.

Peng, Q., K. Li, et al. (2009). "Dendritic cell function in allostimulation is modulated by C5aR signaling." J Immunol **183**(10): 6058-6068.

Perez-Garcia, A., S. Brunet, et al. (2009). "CTLA-4 genotype and relapse incidence in patients with acute myeloid leukemia in first complete remission after induction chemotherapy." Leukemia **23**(3): 486-491.

Pillemer, L., L. Blum, et al. (1954). "The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena." Science **120**(3112): 279-285.

Pio, R., L. Corrales, et al. (2014). "The role of complement in tumor growth." Adv Exp Med Biol **772**: 229-262.

Pishko, E. J., G. S. Kirimanjeswara, et al. (2004). "Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3." European Journal of Immunology **34**(1): 184-193.

Pittari, G., G. Fregni, et al. (2010). "Early evaluation of natural killer activity in post-transplant acute myeloid leukemia patients." Bone Marrow Transplant **45**(5): 862-871.

Podack, E. R. and H. J. Muller-Eberhard (1979). "Isolation of human S-protein, an inhibitor of the membrane attack complex of complement." J Biol Chem **254**(19): 9808-9814.

Podack, E. R. and J. Tschopp (1982). "Polymerization of the ninth component of complement (C9): formation of poly(C9) with a tubular ultrastructure resembling the membrane attack complex of complement." Proc Natl Acad Sci U S A **79**(2): 574-578.

Preissner, K. T., E. R. Podack, et al. (1985). "The membrane attack complex of complement: relation of C7 to the metastable membrane binding site of the intermediate complex C5b-7." Journal of Immunology **135**(1): 445-451.

Profumo, A., R. Mangerini, et al. (2013). "Complement C3f serum levels may predict breast cancer risk in women with gross cystic disease of the breast." J Proteomics **85**: 44-52.

Qazilbash MH, W. E., Rios R, Lu S, Kant S, Giralt S, Estey EH, Thall P, de Lima M, Couriel D, Champlin RE (2004). "Vaccination with the PR1 leukemia-associated antigen can induce complete remission in patients with myeloid leukemia (abstract)." Blood **10**(4): 259.

Quattrin, T., C. H. Albini, et al. (1992). "Urinary excretion of IGF-I and growth hormone in children with IDDM." Diabetes Care **15**(4): 490-494.

Ragusa, M., G. Avola, et al. (2010). "Expression profile and specific network features of the apoptotic machinery explain relapse of acute myeloid leukemia after chemotherapy." BMC Cancer **10**: 377.

Raitanen, M. P., T. Marttila, et al. (2001). "Human complement factor H related protein test for monitoring bladder cancer." J Urol **165**(2): 374-377.

Rashidi, A. and J. F. DiPersio (2016). "Targeting the leukemia-stroma interaction in acute myeloid leukemia: rationale and latest evidence." Ther Adv Hematol **7**(1): 40-51.

Rech, A. J., R. Mick, et al. (2010). "Homeostasis of peripheral FoxP3(+) CD4 (+) regulatory T cells in patients with early and late stage breast cancer." Cancer Immunol Immunother **59**(4): 599-607.

Reis, E. S., R. A. DeAngelis, et al. (2015). "Therapeutic C3 inhibitor Cp40 abrogates complement activation induced by modern hemodialysis filters." Immunobiology **220**(4): 476-482.

Reiter, Y., A. Ciobotariu, et al. (1992). "Sublytic Complement Attack Protects Tumor-Cells from Lytic Doses of Antibody and Complement." European Journal of Immunology **22**(5): 1207-1213.

Reiter, Y., A. Ciobotariu, et al. (1995). "Complement Membrane Attack Complex, Perforin, and Bacterial Exotoxins Induce in K562 Cells Calcium-Dependent Cross-Protection from Lysis." Journal of Immunology **155**(4): 2203-2210.

Reiter, Y. and Z. Fishelson (1992). "Complement membrane attack complexes induce in human leukemic cells rapid expression of large proteins (L-CIP)." Mol Immunol **29**(6): 771-781.

Rescigno, M., F. Avogadri, et al. (2007). "Challenges and prospects of immunotherapy as cancer treatment." Biochim Biophys Acta **1776**(1): 108-123.

Reynes, M., J. P. Aubert, et al. (1985). "Human follicular dendritic cells express CR1, CR2, and CR3 complement receptor antigens." Journal of Immunology **135**(4): 2687-2694.

Riccioni, R., L. Pasquini, et al. (2005). "TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL." Haematologica **90**(5): 612-624.

Ricklin, D., G. Hajishengallis, et al. (2010). "Complement: a key system for immune surveillance and homeostasis." Nat Immunol **11**(9): 785-797.

Ricklin, D. and J. D. Lambris (2013). "Complement in immune and inflammatory disorders: therapeutic interventions." Journal of Immunology **190**(8): 3839-3847.

Riddell, S. R., M. Murata, et al. (2002). "T-cell therapy of leukemia." Cancer Control **9**(2): 114-122.

Riedemann, N. C., R. F. Guo, et al. (2003). "Regulation by C5a of neutrophil activation during sepsis." Immunity **19**(2): 193-202.

Risitano, A. M., R. Notaro, et al. (2010). "Paroxysmal nocturnal hemoglobinuria--hemolysis before and after eculizumab." N Engl J Med **363**(23): 2270-2272.

Ritis, K., M. Doumas, et al. (2006). "A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways." J Immunol **177**(7): 4794-4802.

Rittirsch, D., M. A. Flierl, et al. (2008). "Functional roles for C5a receptors in sepsis." Nat Med **14**(5): 551-557.

Roddie, H., M. Klammer, et al. (2006). "Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia." Br J Haematol **133**(2): 152-157.

Romero, A. I., F. B. Thoren, et al. (2009). "Post-consolidation immunotherapy with histamine dihydrochloride and interleukin-2 in AML." Scand J Immunol **70**(3): 194-205.

Roozendaal, R. and M. C. Carroll (2007). "Complement receptors CD21 and CD35 in humoral immunity." Immunol Rev **219**: 157-166.

Rosenberg, S. A. (2004). "Shedding light on immunotherapy for cancer." New England Journal of Medicine **350**(14): 1461-1463.

Ruggeri, L., M. Capanni, et al. (1999). "Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation." Blood **94**(1): 333-339.

Ruggeri, L., M. Capanni, et al. (2002). "Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants." Science **295**(5562): 2097-2100.

Ruggeri, L., A. Mancusi, et al. (2007). "Natural killer cell alloreactivity in allogeneic hematopoietic transplantation." Curr Opin Oncol **19**(2): 142-147.

Rus, H., F. Niculescu, et al. (1997). "Terminal complement complexes induce cell cycle entry in oligodendrocytes through mitogen activated protein kinase pathway." Immunopharmacology **38**(1-2): 177-187.

Rushmere, N. K., J. M. Knowlden, et al. (2004). "Analysis of the level of mRNA expression of the membrane regulators of complement, CD59, CD55 and CD46, in breast cancer." Int J Cancer **108**(6): 930-936.

Russell, N. H., L. Kjeldsen, et al. (2015). "A comparative assessment of the curative potential of reduced intensity allografts in acute myeloid leukaemia." Leukemia **29**(7): 1478-1484.

Rutkowski, M. J., M. E. Sughrue, et al. (2010). "Cancer and the complement cascade." Mol Cancer Res **8**(11): 1453-1465.

Sacks, S. H. (2010). "Complement fragments C3a and C5a: the salt and pepper of the immune response." Eur J Immunol **40**(3): 668-670.

Sacks, S. H. and W. Zhou (2012). "The role of complement in the early immune response to transplantation." Nat Rev Immunol **12**(6): 431-442.

Sakaguchi, S., T. Yamaguchi, et al. (2008). "Regulatory T cells and immune tolerance." Cell **133**(5): 775-787.

Salama, P., M. Phillips, et al. (2009). "Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer." J Clin Oncol **27**(2): 186-192.

Salih, H. R., H. Antropius, et al. (2003). "Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia." Blood **102**(4): 1389-1396.

Sander, F. E., A. Rydstrom, et al. (2016). "Dynamics of cytotoxic T cell subsets during immunotherapy predicts outcome in acute myeloid leukemia." Oncotarget **7**(7): 7586-7596.

Santegoets, S. J., E. M. Dijkgraaf, et al. (2015). "Monitoring regulatory T cells in clinical samples: consensus on an essential marker set and gating strategy for regulatory T cell analysis by flow cytometry." Cancer Immunol Immunother **64**(10): 1271-1286.

Sauer, M. G., M. E. Ericson, et al. (2004). "A novel system for simultaneous in vivo tracking and biological assessment of leukemia cells and ex vivo generated leukemia-reactive cytotoxic T cells." Cancer Research **64**(11): 3914-3921.

Sayegh, E. T., O. Bloch, et al. (2014). "Complement anaphylatoxins as immune regulators in cancer." Cancer Med **3**(4): 747-758.

Scheffer, S. R., H. Nave, et al. (2003). "Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response in vivo." Int J Cancer **103**(2): 205-211.

Scheibenbogen, C., A. Letsch, et al. (2002). "CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia." Blood **100**(6): 2132-2137.

Schimmer, A. D., I. M. Pedersen, et al. (2003). "Functional blocks in caspase activation pathways are common in leukemia and predict patient response to induction chemotherapy." Cancer Res **63**(6): 1242-1248.

Schlesinger, M., I. Broman, et al. (1996). "The complement system is defective in chronic lymphatic leukemia patients and in their healthy relatives." Leukemia **10**(9): 1509-1513.

Schmid, C., M. Labopin, et al. (2012). "Treatment, risk factors, and outcome of adults with relapsed AML after reduced intensity conditioning for allogeneic stem cell transplantation." Blood **119**(6): 1599-1606.

Schmidt, B., M. Valay, et al. (1995). "Complement Synthesis Influencing Factors Produced by Acute Myeloid Leukemia Blast Cells." Pathol Oncol Res **1**(1): 54-59.

Schoch, C., W. Kern, et al. (2004). "The influence of age on prognosis of de novo acute myeloid leukemia differs according to cytogenetic subgroups." Haematologica **89**(9): 1082-1090.

Schreiber, R. D., L. J. Old, et al. (2011). "Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion." Science **331**(6024): 1565-1570.

Schumacher, W. A., J. C. Fantone, et al. (1991). "The anaphylatoxins C3a and C5a are vasodilators in the canine coronary vasculature in vitro and in vivo." Agents Actions **34**(3-4): 345-349.

Scola, A. M., K. O. Johswich, et al. (2009). "The human complement fragment receptor, C5L2, is a recycling decoy receptor." Molecular Immunology **46**(6): 1149-1162.

Serafini, P., I. Borrello, et al. (2006). "Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression." Semin Cancer Biol **16**(1): 53-65.

Serafini, P., C. De Santo, et al. (2004). "Derangement of immune responses by myeloid suppressor cells." Cancer Immunol Immunother **53**(2): 64-72.

Shedlock, D. J. and H. Shen (2003). "Requirement for CD4 T cell help in generating functional CD8 T cell memory." Science **300**(5617): 337-339.

Shevach, E. M. (2006). "From vanilla to 28 flavors: multiple varieties of T regulatory cells." Immunity **25**(2): 195-201.

Shimoni, A., I. Hardan, et al. (2006). "Allogeneic hematopoietic stem-cell transplantation in AML and MDS using myeloablative versus reduced-intensity conditioning: the role of dose intensity." Leukemia **20**(2): 322-328.

Shushakova, N., J. Skokowa, et al. (2002). "C5a anaphylatoxin is a major regulator of activating versus inhibitory FcgammaRs in immune complex-induced lung disease." J Clin Invest **110**(12): 1823-1830.

Sica, A. and V. Bronte (2007). "Altered macrophage differentiation and immune dysfunction in tumor development." J Clin Invest **117**(5): 1155-1166.

Sier, C. F., K. A. Gelderman, et al. (2004). "Beta-glucan enhanced killing of renal cell carcinoma micrometastases by monoclonal antibody G250 directed complement activation." International Journal of Cancer **109**(6): 900-908.

Silasi-Mansat, R., H. Zhu, et al. (2010). "Complement inhibition decreases the procoagulant response and confers organ protection in a baboon model of Escherichia coli sepsis." Blood **116**(6): 1002-1010.

Sim, R. B., A. J. Day, et al. (1993). "Complement factor I and cofactors in control of complement system convertase enzymes." Methods Enzymol **223**: 13-35.

Sim, R. B. and K. B. Reid (1991). "C1: molecular interactions with activating systems." Immunol Today **12**(9): 307-311.

Sinha, P., V. K. Clements, et al. (2007). "Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response." Journal of Immunology **179**(2): 977-983.

Smyth, M. J., G. P. Dunn, et al. (2006). "Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity." Adv Immunol **90**: 1-50.

Srivastava, R. M., S. C. Lee, et al. (2013). "Cetuximab-activated natural killer and dendritic cells collaborate to trigger tumor antigen-specific T-cell immunity in head and neck cancer patients." Clin Cancer Res **19**(7): 1858-1872.

Stanton, R. J., V. Prod'homme, et al. (2014). "HCMV pUL135 remodels the actin cytoskeleton to impair immune recognition of infected cells." Cell Host Microbe **16**(2): 201-214.

Statistics, O. o. N. (2013). Cancer Statistic Registrations, England. MB1 No. 44. ONS.gov.uk, Office of National Statistics. **2015**.

Stegall, M. D., T. Diwan, et al. (2011). "Terminal complement inhibition decreases antibody-mediated rejection in sensitized renal transplant recipients." Am J Transplant **11**(11): 2405-2413.

Stein EM, S. A., Walter RB et al (2014). Interim analysis of a phase 1 trial of SGN-CD33A in patients with CD33-positive acute myeloid leukaemia (abstract). American Society of Haematology. San Francisco, ASH. **124. Abstract 623**.

Stewart, J. L., J. B. Monahan, et al. (1984). "Measurement of the ratio of the eighth and ninth components of human complement on complement-lysed membranes." Biochemistry **23**(18): 4016-4022.

Strainic, M. G., J. Liu, et al. (2008). "Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells." Immunity **28**(3): 425-435.

Strauss, L., C. Bergmann, et al. (2007). "The frequency and suppressor function of CD4+CD25highFoxp3+ T cells in the circulation of patients with squamous cell carcinoma of the head and neck." Clin Cancer Res **13**(21): 6301-6311.

Sureda, A., P. Bader, et al. (2015). "Indications for allo- and auto-SCT for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2015." Bone Marrow Transplant **50**(8): 1037-1056.

Suzuki, K. G., T. K. Fujiwara, et al. (2007). "GPI-anchored receptor clusters transiently recruit Lyn and G alpha for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1." J Cell Biol **177**(4): 717-730.

Swann, J. B. and M. J. Smyth (2007). "Immune surveillance of tumors." J Clin Invest **117**(5): 1137-1146.

Swerdlow, S. H. (2008). WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon, International Agency for Research on Cancer.

Szczepanski, M. J., M. Szajnik, et al. (2009). "Increased frequency and suppression by regulatory T cells in patients with acute myelogenous leukemia." Clin Cancer Res **15**(10): 3325-3332.

Szczepanski, M. J., M. Szajnik, et al. (2010). "Interleukin-15 enhances natural killer cell cytotoxicity in patients with acute myeloid leukemia by upregulating the activating NK cell receptors." Cancer Immunol Immunother **59**(1): 73-79.

Tack, B. D. and J. W. Prahl (1976). "Third component of human complement: purification from plasma and physicochemical characterization." Biochemistry **15**(20): 4513-4521.

Tajima, F., T. Kawatani, et al. (1996). "Natural killer cell activity and cytokine production as prognostic factors in adult acute leukemia." Leukemia **10**(3): 478-482.

Takabayashi, T., E. Vannier, et al. (1996). "A new biologic role for C3a and C3a desArg: regulation of TNF-alpha and IL-1 beta synthesis." J Immunol **156**(9): 3455-3460.

Takafuji, S., A. Ishida, et al. (2003). "Matrix metalloproteinase-9 release from human leukocytes." J Investig Allergol Clin Immunol **13**(1): 50-55.

Tallman, M. S., G. W. Dewald, et al. (2007). "Impact of cytogenetics on outcome of matched unrelated donor hematopoietic stem cell transplantation for acute myeloid leukemia in first or second complete remission." Blood **110**(1): 409-417.

Tang, Q. and J. A. Bluestone (2008). "The Foxp3+ regulatory T cell: a jack of all trades, master of regulation." Nature Immunology **9**(3): 239-244.

Tarantino, L. M., P. F. Sullivan, et al. (2011). "Using animal models to disentangle the role of genetic, epigenetic, and environmental influences on behavioral outcomes associated with maternal anxiety and depression." Front Psychiatry **2**: 44.

Tettamanti, S., C. F. Magnani, et al. (2013). "Acute myeloid leukemia and novel biological treatments: monoclonal antibodies and cell-based gene-modified immune effectors." Immunol Lett **155**(1-2): 43-46.

Thiel, S. (2007). "Complement activating soluble pattern recognition molecules with collagen-like regions, mannan-binding lectin, ficolins and associated proteins." Molecular Immunology **44**(16): 3875-3888.

Thompson, R. H., M. D. Gillett, et al. (2004). "Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target." Proc Natl Acad Sci U S A **101**(49): 17174-17179.

Thornton, B. P., V. Vetvicka, et al. (1996). "Function of C3 in a humoral response: iC3b/C3dg bound to an immune complex generated with natural antibody and a primary antigen promotes antigen uptake and the expression of co-stimulatory molecules by all B cells, but only stimulates immunoglobulin synthesis by antigen-specific B cells." Clin Exp Immunol **104**(3): 531-537.

Topalian, S. L., C. G. Drake, et al. (2015). "Immune checkpoint blockade: a common denominator approach to cancer therapy." Cancer Cell **27**(4): 450-461.

Topalian, S. L., F. S. Hodi, et al. (2012). "Safety, activity, and immune correlates of anti-PD-1 antibody in cancer." N Engl J Med **366**(26): 2443-2454.

Tourneur, L., S. Delluc, et al. (2004). "Absence or low expression of fas-associated protein with death domain in acute myeloid leukemia cells predicts resistance to chemotherapy and poor outcome." Cancer Res **64**(21): 8101-8108.

Triantafilou, K., T. R. Hughes, et al. (2013). "The complement membrane attack complex triggers intracellular Ca2+ fluxes leading to NLRP3 inflammasome activation." J Cell Sci **126**(Pt 13): 2903-2913.

Trof, R. J., A. Beishuizen, et al. (2007). "Spontaneous remission of acute myeloid leukaemia after recovery from sepsis." Neth J Med **65**(7): 259-262.

Trougakos, I. P. and E. S. Gonos (2002). "Clusterin/apolipoprotein J in human aging and cancer." Int J Biochem Cell Biol **34**(11): 1430-1448.

Trouw, L. A., A. M. Blom, et al. (2008). "Role of complement and complement regulators in the removal of apoptotic cells." Mol Immunol **45**(5): 1199-1207.

Tschopp, J., E. R. Podack, et al. (1982). "Ultrastructure of the membrane attack complex of complement: detection of the tetramolecular C9-polymerizing complex C5b-8." Proc Natl Acad Sci U S A **79**(23): 7474-7478.

Turner, R. T. O. (2010). The Role of Complement in Tumour Formation: Friend or Foe. Department of Infection, Immunity and Biochemistry. Cardiff, Cardiff University. **PhD** 314.

Tzankov, A., C. Meier, et al. (2008). "Correlation of high numbers of intratumoral FOXP3+ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma." Haematologica **93**(2): 193-200.

Uchida, J., Y. Hamaguchi, et al. (2004). "The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy." J Exp Med **199**(12): 1659-1669.

Ustun, C., J. S. Miller, et al. (2011). "Regulatory T cells in acute myelogenous leukemia: is it time for immunomodulation?" Blood **118**(19): 5084-5095.

Ustun, C., A. C. Wiseman, et al. (2013). "Achieving stringent CR is essential before reduced-intensity conditioning allogeneic hematopoietic cell transplantation in AML." Bone Marrow Transplant **48**(11): 1415-1420.

Vadrevu, S. K., N. K. Chintala, et al. (2014). "Complement c5a receptor facilitates cancer metastasis by altering T-cell responses in the metastatic niche." Cancer Research **74**(13): 3454-3465.

Vajdic, C. M. and M. T. van Leeuwen (2009). "What types of cancers are associated with immune suppression in HIV? Lessons from solid organ transplant recipients." Curr Opin HIV AIDS **4**(1): 35-41.

van Lookeren Campagne, M., C. Wiesmann, et al. (2007). "Macrophage complement receptors and pathogen clearance." Cell Microbiol **9**(9): 2095-2102.

Van Tendeloo, V. F., A. Van de Velde, et al. (2010). "Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination." Proc Natl Acad Sci U S A **107**(31): 13824-13829.

Varga, L., E. Czink, et al. (1995). "Low activity of the classical complement pathway predicts short survival of patients with chronic lymphocytic leukaemia." Clinical and Experimental Immunology **99**(1): 112-116.

Verheyden, S., M. Bernier, et al. (2004). "Identification of natural killer cell receptor phenotypes associated with leukemia." Leukemia **18**(12): 2002-2007.

Verheyden, S. and C. Demanet (2008). "NK cell receptors and their ligands in leukemia." Leukemia **22**(2): 249-257.

Vignali, D. (2008). "How many mechanisms do regulatory T cells need?" European Journal of Immunology **38**(4): 908-911.

Vik, D. P. and D. T. Fearon (1985). "Neutrophils express a receptor for iC3b, C3dg, and C3d that is distinct from CR1, CR2, and CR3." Journal of Immunology **134**(4): 2571-2579.

Villanueva, J., A. J. Martorella, et al. (2006). "Serum peptidome patterns that distinguish metastatic thyroid carcinoma from cancer-free controls are unbiased by gender and age." Mol Cell Proteomics **5**(10): 1840-1852.

Vogel, C. W., C. A. Smith, et al. (1984). "Cobra venom factor: structural homology with the third component of human complement." J Immunol **133**(6): 3235-3241.

Vogel, C. W., C. A. Smith, et al. (1984). "Cobra venom factor: structural homology with the third component of human complement." Journal of Immunology **133**(6): 3235-3241.

Volanakis, J. E. and S. V. Narayana (1996). "Complement factor D, a novel serine protease." Protein Sci **5**(4): 553-564.

Volovitz, I., Y. Marmor, et al. (2011). "Split immunity: immune inhibition of rat gliomas by subcutaneous exposure to unmodified live tumor cells." J Immunol **187**(10): 5452-5462.

Walport, M. and P. Lachmann (1984). "C3 receptors, complement deficiency and SLE." Br J Rheumatol **23**(1): 3-5.

Walport, M. J. (2001). "Complement. First of two parts." N Engl J Med **344**(14): 1058-1066.

Walport, M. J. (2001). "Complement. Second of two parts." N Engl J Med **344**(15): 1140-1144.

Walunas, T. L., D. J. Lenschow, et al. (1994). "CTLA-4 can function as a negative regulator of T cell activation." Immunity **1**(5): 405-413.

Wang, S. Y., E. Racila, et al. (2008). "NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement." Blood **111**(3): 1456-1463.

Wang, S. Y., S. Veeramani, et al. (2009). "Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model." Blood **114**(26): 5322-5330.

Wang, X., J. Zheng, et al. (2005). "Increased population of CD4(+)CD25(high), regulatory T cells with their higher apoptotic and proliferating status in peripheral blood of acute myeloid leukemia patients." Eur J Haematol **75**(6): 468-476.

Watson, N. F., L. G. Durrant, et al. (2006). "Expression of the membrane complement regulatory protein CD59 (protectin) is associated with reduced survival in colorectal cancer patients." Cancer Immunol Immunother **55**(8): 973-980.

Weiden, P. L., N. Flournoy, et al. (1979). "Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts." N Engl J Med **300**(19): 1068-1073.

Whiteside, T. L. (2012). "What are regulatory T cells (Treg) regulating in cancer and why?" Semin Cancer Biol **22**(4): 327-334.

Whiteside, T. L., P. Schuler, et al. (2012). "Induced and natural regulatory T cells in human cancer." Expert Opin Biol Ther **12**(10): 1383-1397.

Wilczek, E., R. Rzepko, et al. (2008). "The possible role of factor H in colon cancer resistance to complement attack." International Journal of Cancer **122**(9): 2030-2037.

Wilson, J. G., T. F. Tedder, et al. (1983). "Characterization of human T lymphocytes that express the C3b receptor." Journal of Immunology **131**(2): 684-689.

Winkler, I. G., V. Barbier, et al. (2012). "Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance." Nat Med **18**(11): 1651-1657.

Wolf, A. M., D. Wolf, et al. (2003). "Increase of regulatory T cells in the peripheral blood of cancer patients." Clin Cancer Res **9**(2): 606-612.

Wolf, D., A. M. Wolf, et al. (2005). "The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer." Clin Cancer Res **11**(23): 8326-8331.

Wu, M. C., F. H. Brennan, et al. (2013). "The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion injuries by inhibiting neutrophil mobilization." Proc Natl Acad Sci U S A **110**(23): 9439-9444.

Xu, C., M. Jung, et al. (2005). "Increased CD59 protein expression predicts a PSA relapse in patients after radical prostatectomy." Prostate **62**(3): 224-232.

Xu, S., X. Li, et al. (2015). "Prognostic Value of CD11b Expression Level for Acute Myeloid Leukemia Patients: A Meta-Analysis." PLoS One **10**(8): e0135981.

Xu, W., K. R. Miao, et al. (2011). "Enhancing the action of rituximab by adding fresh frozen plasma for the treatment of fludarabine refractory chronic lymphocytic leukemia." International Journal of Cancer **128**(9): 2192-2201.

Yamaguchi, T., J. B. Wing, et al. (2011). "Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions." Semin Immunol **23**(6): 424-430.

Yamakawa, M., K. Yamada, et al. (1994). "Protection of thyroid cancer cells by complement-regulatory factors." Cancer **73**(11): 2808-2817.

Yang, L. P. and C. M. Perry (2011). "Histamine dihydrochloride: in the management of acute myeloid leukaemia." Drugs **71**(1): 109-122.

Yates, J. W., H. J. Wallace, Jr., et al. (1973). "Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia." Cancer Chemother Rep **57**(4): 485-488.

Young, M. R., M. Newby, et al. (1987). "Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic Lewis lung carcinoma tumors." Cancer Research **47**(1): 100-105.

Ytting, H., I. J. Christensen, et al. (2005). "Serum mannan-binding lectin-associated serine protease 2 levels in colorectal cancer: relation to recurrence and mortality." Clin Cancer Res **11**(4): 1441-1446.

Ytting, H., J. C. Jensenius, et al. (2004). "Increased activity of the mannan-binding lectin complement activation pathway in patients with colorectal cancer." Scand J Gastroenterol **39**(7): 674-679.

Yuan, C., G. Song, et al. (2012). "The characterization and role of leukemia cell-derived dendritic cells in immunotherapy for leukemic diseases." Intractable Rare Dis Res **1**(2): 53-65.

Zhang, L., X. Chen, et al. (2013). "CD40 ligation reverses T cell tolerance in acute myeloid leukemia." J Clin Invest **123**(5): 1999-2010.

Zhang, L., T. F. Gajewski, et al. (2009). "PD-1/PD-L1 interactions inhibit antitumor immune responses in a murine acute myeloid leukemia model." Blood **114**(8): 1545-1552.

Zhang, X., Y. Kimura, et al. (2007). "Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo." Blood **110**(1): 228-236.

Zhong, R. K., M. Loken, et al. (2006). "CTLA-4 blockade by a human MAb enhances the capacity of AML-derived DC to induce T-cell responses against AML cells in an autologous culture system." Cytotherapy **8**(1): 3-12.

Zhou, Q., C. Bucher, et al. (2009). "Depletion of endogenous tumor-associated regulatory T cells improves the efficacy of adoptive cytotoxic T-cell immunotherapy in murine acute myeloid leukemia." Blood **114**(18): 3793-3802.

Zhou, Q., M. E. Munger, et al. (2010). "Program death-1 signaling and regulatory T cells collaborate to resist the function of adoptively transferred cytotoxic T lymphocytes in advanced acute myeloid leukemia." Blood **116**(14): 2484-2493.

Zhou, Q., M. E. Munger, et al. (2011). "Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia." Blood **117**(17): 4501-4510.

Zurlo, J. J., G. P. Schechter, et al. (1989). "Complement abnormalities in multiple myeloma." Am J Med **87**(4): 411-420.

Zwirner, J., O. Gotze, et al. (1998). "The human mast cell line HMC-1 binds and responds to C3a but not C3a(desArg)." Scand J Immunol **47**(1): 19-24.

Immunology and Cell Biology (2012) 00, 1–3

www.nature.com/icb

THEORETICAL ARTICLE

Complement-induced protection: an explanation for the limitations of cell-based tumour immunotherapies

Emma Kempshall, Simon Thebault, B Paul Morgan, Claire L Harris and Awen Gallimore

Complement is involved in the inflammatory response and clearance of infected or altered cells. It is therefore unexpected that complement-deficient animals are less susceptible to carcinogen-induced tumours and more readily control growth of injected tumour cell lines than their wild-type counterparts, implying that complement promotes tumour development and progression. Conversely, natural killer (NK) and CD8$^+$ T cells are known to limit progression of the same tumours. Previous studies indicate that sublytic levels of the complement membrane attack complex protect cells against further attack by lytic doses of complement and other pore-formers such as perforin. We hypothesise that inefficient attack by complement *in vivo* allows tumour cells to avoid lysis by both NK cells and antigen-specific cytotoxic T cells, thereby promoting tumour outgrowth. Complement could thus be limiting the efficacy of NK and T cell-targeted cancer therapies, and the inclusion of complement inhibitors could optimise these immunotherapeutic regimes.
Immunology and Cell Biology advance online publication, 10 July 2012; doi:10.1038/icb.2012.30

Keywords: Complement; induced protection; MAC; immunotherapy; NK; CD8$^+$ T cell; tumour

Chronic inflammation promotes carcinogenesis;[1] however, acute and brisk inflammation can induce spontaneous or treatment-induced tumour regression (reviewed in Schreiber *et al.*[2]). While infection with pathogens leads to danger signals that result in cell clearance, the presence of malignancy appears to result in a chronic, lingering immune response that fails to trigger these danger signals and can promote the neoplastic process while evading immune clearance.

The complement system is a complex pathway comprising >25 serum proteins and cell surface receptors that interact, resulting in a range of functions from direct cell lysis to enhancement of the adaptive immune response.[3] When complement is activated, a cascade mechanism results in the formation of the membrane attack complex (MAC), containing complement proteins C5b, C6, C7, C8 and C9. This is inserted into the plasma membrane of target cells and, at high doses, can result in rapid cell death.[4] Multiple pathways of activation and the catalytic nature of many steps mean that regulation of complement is complex.[4] The host protects autologous cells from inappropriate complement attack by the expression of serum and membrane-bound complement-regulatory proteins (CRegs).

Complement activation and deposition can be detected on tumour cells from a range of malignancies *ex vivo*.[5,6] Neoplastic cells have been shown to synthesise and secrete complement components.[7,8] However, this level of attack appears insufficient to kill cells within the developing tumour, possibly because of the widespread expression of CRegs by cancers,[9,10] resulting in continuous, low level complement activation.

Whether complement pathways have a role in tumour initiation is not clear. However, there is accumulating evidence that they do influence progression of established tumours. Most notably, it was recently shown that tumour progression is accelerated by complement component C5a, as a result of its ability to activate and recruit myeloid-derived suppressor cells.[11] Furthermore, work done in our laboratory has shown that complement-deficient mice or those treated with a complement-blocking antibody had decreased tumour growth.[12] There is also evidence to support a role for CRegs in influencing cancer progression in humans.[13] The precise impact of CReg expression is not fully understood, as some studies have reported that low levels of CReg expression are associated with disease progression,[14–16] while others indicate that high levels of CRegs are associated with decreased survival.[17–20] Despite these seemingly conflicting findings, these data are compatible with a role for complement activation in influencing the phenotype of tumour cells and hence their capacity to proliferate and survive.

COMPLEMENT-INDUCED PROTECTION

It has previously been shown that sublytic levels of MAC protect cells against further attack by fully lytic doses of complement and other pore formers such as perforin.[21,22] The physiological relevance of this *in vitro*-defined process, termed 'induced protection', is unclear, though it has been speculated that it prevents lysis of innocent bystander cells at sites of immune activity, thereby limiting the extent of collateral damage caused by a given immune response.[22] As resistance to perforin is also induced, it is possible that attack of

The Institute of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, UK
Correspondence: Dr E Kempshall, The Institute of Infection and Immunity, Cardiff University, Heath Park Campus, 3rd Floor Henry Wellcome Building, Cardiff CF14 4XN, UK.
Email: Kempshallec1@cardiff.ac.uk
Received 15 March 2012; revised 8 May 2012; accepted 9 May 2012

tumour cells with sublytic MAC also protects against lysis by natural killer (NK) cells and tumour-specific CD8$^+$ T cells, both of which utilise perforin to form the pores through which inducers of cell death, such as granzymes, are introduced to the target cell.

NK cells have been shown to control tumour development in experimental mice.[23] Tumour infiltration by NK cells is associated with a favourable prognosis in several human cancers.[24] There is also evidence that tumours evolve to escape elimination by NK cells,[25] with some having been shown to reversibly downregulate the activity of NK cells.[26] Using mouse models, we have found that while NK cells and CD8$^+$ T cells have a key role in limiting progression of carcinogen-induced tumours, complement actually promotes progression of these same tumours.[12] Antigen-specific lymphocytes can have an important role in tumour immune surveillance.[27] Tumour-infiltrating lymphocytes are, however, often hypo-functional, with T cells specific for tumour antigen appearing to co-exist with the tumour without eliminating it (reviewed in Rosenberg[28]). Although there has been success in adoptively transferring tumour-specific cytotoxic T lymphocytes, the expected anticancer effects have been limited, with the full effector function seen when they are exposed to tumour antigen *in vitro* being progressively lost after a short period of active responses *in vivo*.[28] In a similar way, vaccine-induced T cells can be induced at high frequencies, yet these cells do not result in significant elimination of tumour cells.[28]

NK cells and CD8$^+$ T cells exert their anti-tumour effects not only through cytotoxic mechanisms but also through the release of cytokines, most notably IFNγ. As such, complement-induced protection would not completely inhibit the anti-tumour effects of these effector cells. Despite a wealth of preclinical and clinical data, there is no clear consensus regarding the relative contribution of cytotoxicity and IFNγ to the elimination of tumour cells by NK cells and T cells. What is clear, however, mainly from studies using mouse models, is that both mechanisms are important. A higher incidence of spontaneously arising B-cell lymphomas[29] and carcinogen-induced fibrosarcomas[30] is observed in perforin-deficient animals. These mice are also more susceptible to metastatic disease following injection with tumour cells derived from lung and prostate cancer.[31] It is also the case that perforin makes a significant contribution to elimination of B16 melanoma in mouse models of adoptive T-cell transfer.[32] With these data in mind, it is reasonable to expect that compromising the killing capacity of NK cells and cytotoxic T cells, through a mechanism such as complement-induced protection, would favour tumour progression.

We hypothesise that inefficient attack on tumour cells by sublytic doses of MAC is an *in vivo* mechanism by which malignant cells evade killing by NK cells and antigen-specific cytotoxic T cells, thereby promoting tumour outgrowth (see Figure 1).

TESTING THE HYPOTHESIS

The hypothesis described above could be tested *in vitro* using published methods. It has already been shown that sub-lethally attacked tumour cells become resistant to pore formation by perforin that has been purified to homogeneity from the granules of NK cells.[22] This concept of resistance could be expanded to a 'whole-cell' assay by testing the resistance of tumour cells, which have been subjected to sublytic doses of complement, to killing in conventional NK-cell and CTL-killing assays.

Established mouse models could then be used to assess the *in vivo* effects of complement-induced protection. The role of sublytic complement on tumour-cell killing by CD8$^+$ T cells and NK cells could be explored using a C6-specific blocking antibody, which prevents MAC formation whilst allowing production of C5a. This will allow the effect of sublytic MAC to be differentiated from that of the anaphylotoxin, C5a.

NK cells can be activated in mice, for example, by injection with Poly-ic, a synthetic dsRNA analogue that activates cells through binding to TLR3. Mice treated in this way could be divided into two groups, one of which could be treated with C6-specific blocking

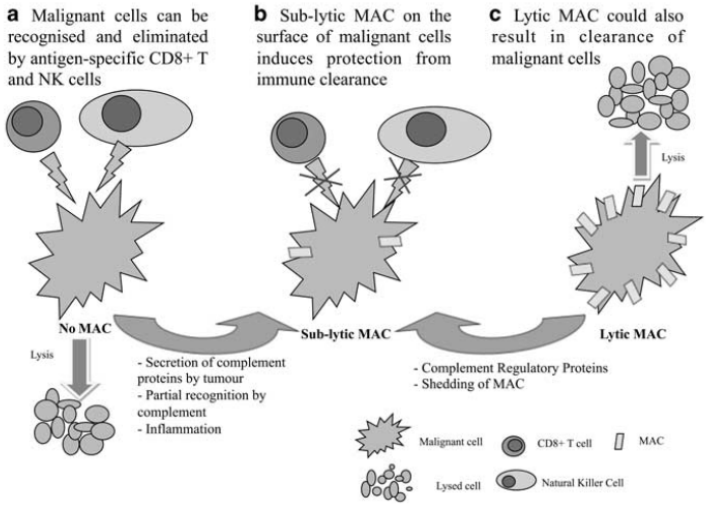


Figure 1 Potential mechanisms resulting in induced protection of malignant cells. Malignant cells use a range of mechanisms including secretion of complement proteins, expression of complement-regulatory proteins and shedding of MAC in order to maintain complement in a constant state of low-level activation. In addition, low-level complement activation may result from malignant cells being partially recognised by the host's immune system. Concomitant low-grade infection or inflammation may also result in the correct conditions required for induced protection to occur. A full colour version of this figure is available at the *Immunology and Cell Biology* journal online.

antibodies and the other with irrelevant control antibodies. Both groups could subsequently be injected intravenously with titrated numbers of RMAS cells (mouse T-cell lymphoma) that express low levels of MHC class I and hence are highly susceptible to NK cell killing.[33] Should sublytic complement compromise the anti-tumour activity of the activated NK cells, lower tumour take will be observed in the C6-specific antibody-treated mice.

Similar methods could then be used to assess the complement's effect on $CD8^+$ T cells. Tumour cell lines engineered to express a surrogate antigen (for example, influenza nucleoprotein) could be used to generate tumours *in vivo*. Nucleoprotein-specific $CD8^+$ T cells, engineered to kill nucleoprotein-expressing tumour cells *in vivo*, could be used as a form of adoptive immunotherapy. Concurrently, C6-specific antibodies could be given to one group of mice and tumour growth compared in this group and those treated with control antibodies. This experiment would help address whether inhibition of sublytic MAC increases the efficacy of adoptively transferred tumour-specific $CD8^+$ T cells.

THE FINAL PROOF?

In vitro and mouse models offer 'proof-of-principle' and will provide evidence on which to base further research into the relevance of complement-induced protection in patients with cancer. Pharmacological blockade of the C5a receptor in mice significantly impairs tumour growth, thus providing evidence of the therapeutic potential of complement inhibition in the treatment of cancer.[11] Thus, the natural extension of the experiments described above is the establishment of pilot clinical studies comprising complement therapies in patients with cancer.

Eculizumab (Soliris; Alexion, Cheshire, CT, USA) is a monoclonal antibody against complement component C5, which inhibits C5 cleavage, thus preventing the formation of MAC. It was the first complement-specific drug approved by the US FDA and is now licensed for use in the UK for the treatment of paroxysmal nocturnal haemoglobinuria. More recently it has also been approved by the FDA for use in atypical haemolytic uraemic syndrome. Eculizumab has been found to be safe and well tolerated and is currently being explored in 28 other trials for a range of conditions.[34] Complement inhibition is an attractive prospect as its utility is not confined to a particular tumour type. It could therefore be taken into clinical trials as an adjunctive treatment for any malignancy, offering the potential to both improve long-term remission following chemotherapy and optimise all NK and T cell-targeted therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Dr Kempshall is supported by Cancer Research UK. Dr Gallimore is funded by a University Award from the Wellcome Trust.

Authors contributions: All authors contributed to the writing of this paper.

1 Mantovani A. Molecular pathways linking inflammation and cancer. *Curr Mol Med* 2010; **10**: 369–373.
2 Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011; **331**: 1565–1570.
3 Walport MJ. Complement. first of two parts. *N Engl J Med* 2001; **344**: 1058–1066.
4 Morgan BP, Meri S. Membrane-proteins that protect against complement lysis. *Springer Semin Immun* 1994; **15**: 369–396.
5 Niculescu F, Rus HG, Retegan M, Vlaicu R. Persistent complement activation on tumor cells in breast cancer. *Am J Pathol* 1992; **140**: 1039–1043.
6 Lucas SD, Karlsson-Parra A, Nilsson B, Grimelius L, Akerstrom G, Rastad J *et al*. Tumor-specific deposition of immunoglobulin G and complement in papillary thyroid carcinoma. *Hum Pathol* 1996; **27**: 1329–1335.
7 Kitano E, Kitamura H. Synthesis of the third component of complement (C3) by human gastric cancer-derived cell lines. *Clin Exp Immunol* 1993; **94**: 273–278.
8 Barnum SR, Ishii Y, Agrawal A, Volanakis JE. Production and interferon-gamma-mediated regulation of complement component C2 and factors B and D by the astroglioma cell line U105-MG. *Biochem J* 1992; **287**(Part 2): 595–601.
9 Fishelson Z, Donin N, Zell S, Schultz S, Kirschfink M. Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. *Mol Immunol* 2003; **40**: 109–123.
10 Gorter A, Meri S. Immune evasion of tumor cells using membrane-bound complement regulatory proteins. *Immunol Today* 1999; **20**: 576–582.
11 Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C *et al*. Modulation of the antitumor immune response by complement. *Nat Immunol* 2008; **9**: 1225–1235.
12 Turner RTO. The role of complement in tumour formation: friend or foe [PhD thesis] Cardiff: Cardiff University, 2010. Available from the Health library, Cardiff University. http://library.cardiff.ac.uk/vwebv/holdingsInfo?bibId=889882.
13 Gancz D, Fishelson Z. Cancer resistance to complement-dependent cytotoxicity (CDC): problem-oriented research and development. *Mol Immunol* 2009; **46**: 2794–2800.
14 Koretz K, Bruderlein S, Henne C, Moller P. Expression of CD59, a complement regulator protein and a second ligand of the CD2 molecule, and CD46 in normal and neoplastic colorectal epithelium. *Br J Cancer* 1993; **68**: 926–931.
15 Hofman P, Hsi BL, Manie S, Fenichel P, Thyss A, Rossi B. High expression of the antigen recognized by the monoclonal antibody GB24 on human breast carcinomas: a preventive mechanism of malignant tumor cells against complement attack? *Breast Cancer Res Treat* 1994; **32**: 213–219.
16 Madjd Z, Durrant LG, Bradley R, Spendlove I, Ellis IO, Pinder SE. Loss of CD55 is associated with aggressive breast tumors. *Clin Cancer Res* 2004; **10**: 2797–2803.
17 Watson NF, Durrant LG, Madjd Z, Ellis IO, Scholefield JH, Spendlove I. Expression of the membrane complement regulatory protein CD59 (protectin) is associated with reduced survival in colorectal cancer patients. *Cancer Immunol Immunother* 2006; **55**: 973–980.
18 Durrant LG, Chapman MA, Buckley DJ, Spendlove I, Robins RA, Armitage NC. Enhanced expression of the complement regulatory protein CD55 predicts a poor prognosis in colorectal cancer patients. *Cancer Immunol Immunother* 2003; **52**: 638–642.
19 Xu L, Shao H, Wu W, Cao Z, Zhao Z, Liu H *et al*. Co-expression of the complement regulatory proteins human DAF and CD59 with an IRES-mediated dicistronic mammalian vector enhances their cell protective effects. *Int J Mol Med* 2005; **16**: 409–414.
20 Xu C, Jung M, Burkhardt M, Stephan C, Schnorr D, Loening S *et al*. Increased CD59 protein expression predicts a PSA relapse in patients after radical prostatectomy. *Prostate* 2005; **62**: 224–232.
21 Reiter Y, Ciobotariu A, Fishelson Z. Sublytic complement attack protects tumor-cells from lytic doses of antibody and complement. *Eur J Immunol* 1992; **22**: 1207–1213.
22 Reiter Y, Ciobotariu A, Jones J, Morgan BP, Fishelson Z. Complement membrane attack complex, perforin, and bacterial exotoxins induce in K562 cells calcium-dependent cross-protection from lysis. *J Immunol* 1995; **155**: 2203–2210.
23 Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. *In vivo* natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci USA* 2000; **97**: 2731–2736.
24 Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C *et al*. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* 1997; **79**: 2320–2328.
25 Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* 2002; **419**: 734–738.
26 Fauriat C, Just-Landi S, Mallet F, Arnoulet C, Sainty D, Olive D *et al*. Deficient expression of NCR in NK cells from acute myeloid leukemia: evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. *Blood* 2007; **109**: 323–330.
27 Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C *et al*. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; **313**: 1960–1964.
28 Rosenberg SA. Shedding light on immunotherapy for cancer. *New Engl J Med* 2004; **350**: 1461–1463.
29 Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J Exp Med* 2000; **192**: 755–760.
30 van den Broek ME, Kagi D, Ossendorp F, Toes R, Vamvakas S, Lutz WK *et al*. Decreased tumor surveillance in perforin-deficient mice. *J Exp Med* 1996; **184**: 1781–1790.
31 Smyth MJ, Thia KY, Cretney E, Kelly JM, Snook MB, Forbes CA *et al*. Perforin is a major contributor to NK cell control of tumor metastasis. *J Immunol* 1999; **162**: 6658–6662.
32 Kline J, Zhang L, Battaglia L, Cohen KS, Gajewski TF. Cellular and molecular requirements for rejection of B16 melanoma in the setting of regulatory T cell depletion and homeostatic proliferation. *J Immunol* 2012; **188**: 2630–2642.
33 van den Broek MF, Kagi D, Zinkernagel RM, Hengartner H. Perforin dependence of natural killer cell-mediated tumor control *in vivo*. *Eur J Immunol* 1995; **25**: 3514–3516.
34 Ricklin D, Lambris JD. Complement-targeted therapeutics. *Nat Biotechnol* 2007; **25**: 1265–1275.