Development of Cancer Immunotherapeutics

Targeting Complement Regulatory Protein CD55

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Contents

Figures

CHAPTER 1: Introduction

CHAPTER 2: Materials and Methods

CHAPTER 3: Development of monoclonal antibodies capable of neutralising CD55

CHAPTER 4: Development and assessment of SCR 1-3 Fc DNA vaccine eliciting antibody responses to CD55

CHAPTER 5: Development of an SCR 1-3 Fc DNA vaccine incorporating heteroclitic epitopes in order to stimulate CTL mediated responses

Abstract

CD55 is one of the complement regulatory/inhibitory proteins and is over-expressed on a wide range of solid tumours. CD55 is also known to be deposited within tumour stroma and is secreted in an active soluble form, mediated by matrix metalloproteinase-7. The complement cascade forms part of the innate immune system and culminates in cell lysis of targeted cells. As a complement regulatory protein, the primary function of CD55 is to accelerate the decay of complement components preventing formation of the membrane attack complex.

CD55 is also known to be a ligand for the T cell early activation antigen CD97, and their interaction has been shown to inhibit the proliferation of activated T cells.

This project aimed to develop anti-tumour immunotherapeutics aimed at exploiting CD55 as a tumour associated antigen. Initial strategies were to develop monoclonal antibodies, specific to identified epitopes from within the CD55 protein sequence, capable of binding, and neutralising CD55's decay accelerating activity. Developed antibodies would also have the potential to induce antibody dependent cell cytotoxicity, thus blocking CD55 protection of tumours and mediating an active anti-tumour response. Antibodies were raised specific to CD55 derived linear peptides, which have been used for the assessment of CD55 expression in breast tumour sections. Monoclonal antibodies failed to recognise natively expressed protein on viable tumour cells and alternate strategies were developed.

An effective immunotherapy for the treatment of cancer would engage both cellular and humoral mediated responses for effective clearance of target cells. In order to achieve this, a DNA vaccine incorporating a human IgG Fc tail was developed expressing the active sites of CD55, containing HLA-A*201 restricted heteroclitic epitopes. The vaccines were used to immunise HLA-A*201 HHDII transgenic mice and CD55 specific responses were assessed. One of the vaccines analysed, elicited CD55 specific antibodies capable of recognising tumour cells *in vitro* and also generated epitope specific $CD8^+$ T cell mediated lysis of epitope bearing cells. The frequency of CD55 specific T cells was obtained via antigen specific IFNγ release

ELISPOT assays and the cytokine profile of responses generated was assessed via luminex analysis.

In conclusion, CD55 remains a viable target for immunotherapies aimed at CD55 bearing tumours. DNA vaccines encoding modified epitopes are capable or raising cellular and humoral responses to this antigen and further studies should be completed in order to determine anti-cancer effects in tumour bearing models.

Abbreviations

AP	Alternative complement activation Pathway
ABTS	2,2'-Azino-bis(3-ethylBenzoThiazoline-6-Sulfonic acid) diammonium salt
ADCC	Antibody Dependent Cell Cytotoxicity
APC	Antigen Presenting Cell
bp	Base Pairs
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CD35	Complement receptor 1
CD46	Membrane cofactor protein
CD55	Decay accelerating factor (DAF)
CD59	Protectin
cDNA	copyDNA
CDR	Complementarity Determining Regions
CEA	Carcinoembryonic Antigen
CFA	Complete Freund's Adjuvant
CP	Classical complement activation Pathway
CSF	Cerebro Spinal Fluid
C1-C9	Complement serum proteins
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic Lymphocytes Associated Antigen 4
DC	Dendritic Cell
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DTH	Delayed Type hypersensivity
ECM	ExtraCellular Matrix
EDTA	EthyleneDiamineTetraAcetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter

FCS	Foetal (Bovine) Calf Serum
FITC	Fluorescein Iso Thiocyanate
FL-1	Fluorescent laser-1
FSC	Forward Scatter
GPI	Glycophosphatidylinositol
HAMA	Human Anti Mouse Antibody
HLA	Human Leukocyte Antigen
HRP	Horse-Radish Peroxidase
IFA	Incomplete Freund's Adjuvant
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin
KIR	Killer Inhibitory Receptor
KLH	Keyhole Limpet Hemocyanin
LPS	LipoPolySaccharide
Mab	Monoclonal Antibody
MAC	Membrane Attack Complex
MCP	Membrane Cofactor Protein
MBL	Mannose Binding Lectin
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
MHC	Major Histocompatibility Complex
MICA	MHC Class I chain related A
MIP1α/CCL3	Macrophage Inflammatory Protein 1α
MIP1β	Macrophage Inflammatory Protein 1β
MMp-7	Matrix Metalloproteinase 7
NCS	Newborn Calf Serum
NHS	Normal Human Serum
NK	Natural Killer Cells
NKT	Natural Killer T cells
o/n	over night
OD	Optical Density
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain reaction
PNH	Paroxysmal Nocturanl Haemoglobinurea
Pro	Proline
PRR	Pattern Recognition Receptor
RNA	RiboNucleic Acid
sCD55	soluble CD55
SCR	Short Consensus Repeat
Ser	Serine
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SSC	Side Scatter
TAP	Transporters Associated with antigen Presentation
Thre	Threonine
TCR	T cell Receptor
Th_1	T cell helper response type 1
Th_2	T cell helper response type 2
TLR	Toll-Like Receptor
TNFα	Tumour Necrosis Factor-alpha
Tween	polyethylene sorbitan monolaurate
UV	Ultra Violet
v/v	Volume per Volume
w/v	Weight per Volume
w/w	Weight per Weight

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Publications Resulting from this Thesis

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Chapter 1: Introduction

1.1: Immunity

Immunity is said to be 'a state of protection from the invasive or pathogenic effects of potential infective microbes, or to the effects of potentially toxic antigenic substances'. The immune system has both specific and non-specific components; acquired or specific immunity utilises the activity of cellular responses encompassing lymphocytes and their products, while the innate defence mechanism provides first line protection until acquired responses can develop.

1.2: Innate Immunity

Generally, most invading microorganisms encountered by the immune system are constitutively recognised, due to the presence of molecules such as lipopolysaccharides (LPS), which have evolved with the human immune system (Hamann et al., 1998). Such organisms are readily cleared by this innate non-adaptive response. However, if the innate system is eluded, specific immunity, which supplements and augments non-specific pathways, produces a more effective total response. It is this comprehensive interaction between the innate arm of the immune system and the adaptive branch that enables complete immune effector functions.

Many pathogens, particularly prokaryotes possess molecular structures which are not shared with 'host' organisms but which are homologous with other related pathogens. These structures are often invariant and are loosely identified as PAMPs or pathogen associated molecular patterns (Blach-Olszewska et al., 2005). Examples include flagellin of bacterial flagella, peptidoglycan of gram-positive bacteria, lipopolysaccharides (LPS) of gram-negative bacteria, double stranded DNA of some viruses and unmethylated DNA. Within the innate immune system many pattern recognition receptors (PRRs) have evolved that constitutively recognise PAMPs and initiate host responses. PRRs can be loosely grouped into three categories, secreted molecules which circulate within the lymphatic and blood systems and phagocytic receptors present on cells such as macrophages that bind pathogens for phagocytosis. The final group of PRRs are cell surface receptors which bind specific PAMPs and initiate signal transduction pathways which lead to the production and release of

effector molecules (cytokines). Many of these innate receptors have been identified and include the Toll-like receptors. More recently, intracellular microbial 'sensors' such as the NOD like receptors (NLRs) and the RIG-like helicases (RLHs) have been identified which survey the cytoplasm for the presence of intracellular invasion (Meylan et. al., 2006)

1.3: Toll Like receptors (TLRs)

A class of PPRs called Toll like receptors (TLRs), reviewed by Underhill and Ozinsky, 2002, recognise pathogens or pathogen derived products and initiate signalling events leading to activation of innate host defences. TLR signalling initiates acute inflammatory responses by the induction of antimicrobial genes and inflammatory cytokines and chemokines (Janeway et. al., 2002, and Takeda et. al., 2003). **Figure 1.1** summarises some of the TLRs currently identified and their associated ligands and responses generated.

Figure 1.1: Toll receptors, ligands and generated responses

PAMP	Pathogen/Stimulus	TLR	Response
Triacyl lipopeptides		TLR1	
Flagellin	Many bacteria	TLR5	Initiates inflammation
ssRNA	Imiquimod	TLR7,TLR8	Stimulates DC maturation
Lipoproteins	Eubacteria	TLR2	Initiates inflammation
CpG	Various microbial pathogens	TLR9	Initiates inflammation
HSP; 60,70 and GP69	Stressed cells	TLR4 (HSP60), TLR2/4 (HSP70 & GP69)	Stress response, protein folding
Fibronectin, Fibrinogen, hearin	Many sources	TLR4	DC maturation, inflammatory-gene induction
dsRNA	RNA virses	TLR3	Immune system recognition and pathogen clearance
Peptidoglycan	Most bacteria	TLR2, CD14 TLR6 forms heterodimer with TLR2	Initiates inflammation (TLR2)/enhancement (TLR4)
Lipoteichoic Acid	Many gram-positive bacteria	TLR2, TLR4	Initiates inflammation

Adapted from: Beg, 2002; Aderem and Ulevitch, 2000; Akira 2003, Pandey and Agrawal 2006.

The primary role of PRRs is to provide immediate protection from invading pathogens, mediated through activation of complement and phagocytosis. In addition, anti- microbial peptides and proteins are induced by TLRs on multiple cell types. TLRs also induce cytokines such as IL-1β and TNF and chemokines that collectively

induce acute inflammatory responses to pathogens. TLRs also induce enhanced phagocytosis mediated by macrophages and induce type I interferons in response to viral DNA or RNA in order to clear viral infections. TLRs expressed by DCs, in response to microbial infection, initiate signalling events that lead to DC maturation. This process up-regulates MHC expression bearing pathogen derived peptides and also expression of the B7 family of co-stimulatory molecules.

TLRs have also been associated with adaptive responses and with tolerance mechanisms. Central tolerance allows for the deletion of thymocytes with high affinity to self antigens within the thymus. Current understanding suggests that while TLRs do not play a significant role in thymic negative selection, it is well established that expression of co-stimulatory molecules on antigen presenting cells is essential for its induction, although it is not currently determined whether TLRs control co-stimulatory molecule induction on thymic APCs (Chandrashekhar et. al., 2004).

1.4: Cells of the Innate Immune System

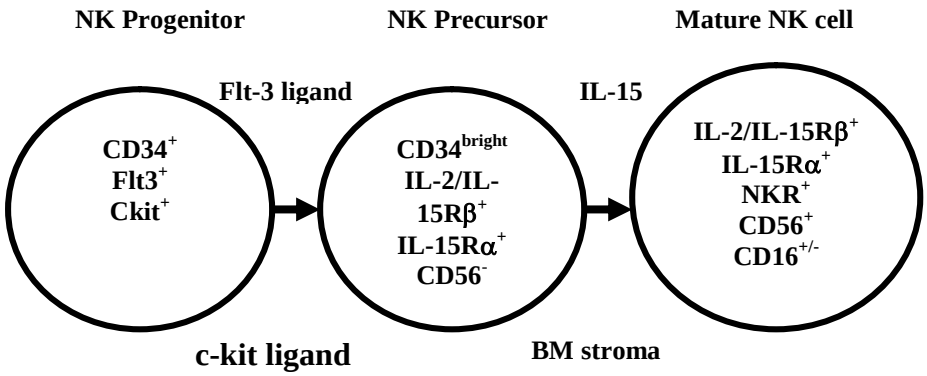
The innate system is composed of a number of specialised cells that recognise and respond to pathogens or cellular stress. These include NK cells, NKT cells and γδ T cells. Antigen presenting cells are innate cells that interact with the acquired immune system in order to form a bridge between the innate and adaptive immune systems.

1.5: NK cells

NK cells are one of the cellular mediators of innate defence and are of lymphoid origin. They can recognise and kill aberrant cells and generate soluble factors, chemokines and cytokines, which have both anti-microbial effects and priming effects on other cells of the immune system.

NK cell progenitors are generated from $CD34^+$ haematopoietic stem cells within the bone marrow and require stromal cell contact for maturation.

Figure 1.2: Maturation of NK cells. Adapted from Colucci et. al., 2003.



Early acting cytokines stimulate progenitor cells to differentiate into NK precursor cells within the bone marrow stroma (**figure 1.2** represents the development from progenitor to mature NK cell). C-kit ligand and Flt-3 ligand directly induce the expression of IL-2Rβ, enabling IL-2 or IL-15 responsiveness committing these precursors to the NK cell lineage (Mrozek et. al., 1996 and Williams et.al., 1997). The heterogeneity of bone marrow immature NK cells is considerable and mature NK cells have been shown to fall into two subsets. In the blood 90% of NK cells are $CD56^{DIM}$ and express high levels of CD16(FCRIII) possessing low proliferative capacity and are identified as 'killers', and 10% are $CD56^{BRIGHT}$ with low CD16 levels (Robertson and Ritz, 1990), being identified as cytokine producers.

NK cell activity is mediated by a variety of cytokines by possessing multiple cytokine receptors as well as by expressing both cytokines and chemokines themselves. IL-15 is prominent in NK cell activity, by driving maturation, proliferation, cytotoxicity and promoting further cytokine production (Fehniger and Caligiuri, 2001). IL-15 alone drives GM-CSF production while in concert with IL-12, drives NK cells to produce IFNγ and TNFα (Carson, Giri and Lindemann, 1994). These cytokines also act synergistically to promote MIP-1α/CCL3 (macrophage inflammatory protein) and MIP-1β/CCL4 (Fehniger et. al., 1999 and 2002).

Developing NK cells acquire a complex repertoire of activating and inhibitory receptors, determining responses through a fine balance between activation and inhibition (Borrego et. al., 2002).

The main mechanism for NK cell mediated cytolysis is dependent on perforin and granzyme activity although alternate mechanisms for inducing lysis have been identified, mediated through the role of FASL and tumour-necrosis factor related apoptosis inducing ligand (TRAIL) dependent receptors (Smyth et. al., 2002).
The main groups of identified NK cell receptors can be divided into three main families, namely the killer immunoglobulin-like receptors (KIRs), immunoglobulin like transcripts (ILTs) and C-type lectin receptors. KIRs are surface inhibitory receptors specific for allelic forms of HLA class I molecules expressed by NK and T cell subsets and are reviewed by Moretta et al., (2004). Upon engagement with their respective class I molecules, KIRs block NK cell activation and function. KIR sequences are highly polymorphic and haplotypes are shown to vary in the number of genes they contain (Moretta et al., 2004). **Figure 1.3** shows a brief summary of many of the currently identified NK cell receptors.

Figure 1.3: Summary of some of the diverse range of characterised NK cell inhibitory and activating receptors.

Inhibitory			Activating		
Receptor	**Structural type**	**Ligand**	**Receptor**	**Structural type**	**Ligand**
KIR2DL	Ig	HLA-C	KIR2DS	Ig	HLA-C
KIR3DL	Ig	HLA-B,C	KIR3DS	Ig	HLA-B
LILRB1,2	Ig	HLA class I	LILRA3	Ig	Unknown
CD94:NKG2A	Lectin	HLA-E	CD94:NKG2C/E	Lectin	HLA-E
LAIR-1	Ig	Unknown	LAIR-2	Ig	Unknown
			NKG2D	Lectin	MIC-A,B & others
			NKp30	Ig	Unknown
			NKp44	Ig	Unknown
			NKp46	Ig	Unknown
			CD16	Ig	Fc

Adapted from Janeway et. al., The Immune System, 2005. Receptors organised according to signalling potential, structural type and associated ligands. KIR nomenclature, 2DL and 3DL correspond to rceptors possessing long cytoplasmic tails and two or three immunoglobulin-like domains. 3DS corresponds to receptors possessing short cytoplasmic tails and 3 Ig-like domains.

Whereas all KIRs and leukocyte associated immunoglobulin like receptors (LAIRs) are expressed by NK cells, only a few of the leukocyte immunoglobulin like receptors (LILRs) are expressed by NK cells. NKp30, NKp44 and NKp46 are collectively known as the natural cytotoxicity receptors, which are important for tumour cell killing and are exclusively expressed by NK cells (Moretta et. al., 2000).

NK cells also mediate antibody dependent cellular cytotoxicity through an Fc receptor complex (CD16) which enables NK cells to engage in the elimination of antibody targeted cells.

This varied combination of stimulatory responses produced through multiple co-stimulatory receptors; shape the overall immune response, dependent upon the nature of the environment created by infections. While there is limited evidence to support this, the presence of NKG2D found on NK, γδT and $CD8^+$ T cells binds the stress induced ligand MICA, which reduces the TCR activation threshold, making NKG2D a co-stimulatory molecule effective at the tissue site (Groh et al., 2001).

1.6: NKT cells

NKT lymphocytes were originally characterised in mice as cells expressing both a T cell antigen receptor (TCR) and NK1.1 (NKR-P1 or CD161c, a C-lectin type NK receptor (Bendelac et. al., 1997). More recently however, NKT cells have been defined as cell that almost always have an invariant Vα 14-Jα 18 rearrangement and reactivity to the glycophospholipid α-galactosylceramide (αGalCer) when presented by the class I-like molecule CD1d (Godfrey et. al.,2004). NKT cells arise in the thymus and their positive selection is mediated by CD1d expressing bone marrow-derived cells. NKT cells development also requires the expression of NFκB1 (Sivakumar et. al.,2003 and Stanic et. al., 2004) and the expression of the gene for the inhibitor of κB kinase, *Ikk2* (Schmidt-Supprian et. al.,2004). Townsend et. al., 2004 showed that differentiation of NKT cells requires the transcription factor T-bet (T-box expressed in T cells), a factor identified for the induction of IFNγ synthesis and Th1 immunity in several cell types. NKT cells form a mature, immune competent population capable of producing IL-4 and IFNγ immediately following TCR stimulation (Stetson et. al., 2003). The typical response of NKT cells is the rapid and

copious production of cytokines, particularly Th1 and Th2 cytokines as mentioned earlier. However, TCR activated NKT cells produce many other cytokines including IL-2, tumour necrosis factor (TNF), IL-5, IL-13 and GM-CSF. This mixture of responses opens several questions as to how a regulated response is generated and evidence favours several explanations which are not mutually exclusive. One possibility is that the quality of the TCR signal influences the profile produced, by analogy with the effects of altered peptide ligands on $CD4^+$ T cells. This is supported by OCH, an αGalCer analog reportedly stimulated a higher ratio of IL-4 to IFNγ secretion when added to total spleen cultures (Miyamoto et. al., 2001 and Stanic et. al., 2003). In contrast, C-glycoside analog of αGalCer reportedly stimulates a higher ratio of IFNγ to IL-4. Cytokine production resulting from TCR stimulation is also not influenced by IL-4, IL-12 or other factors that influence cytokine production of conventional T cells (Kronenberg, 2005). Another possibility is that integration of signals from different types of receptors influence the pattern of cytokines produced by NKT cells. This is supported as although IL-12 is not required for NKT cell IFNγ production following αGalCer stimulation, IL-12 can stimulate IFNγ production in the absence of αGalCer (Leite-De Moraes et. al., 1999 and 1998). Cross linking of NK1.1 has also been shown to achieve this. Most ligands identified as being associated with NKT cells recognise CD1d in conjunction with hydrophobic ligands, namely glycolipids (Burden et. al., 1999 and Benlagha et. al.,2000).

NKT cells also respond to tumour derived lipid extracts, including phospholipids, in the context of CD1d which suggests the presence of a natural lipid ligand, possibly altered within the tumour tissue (Gumperz et. al., 2000). Numerous sources identify that NKT cells recognise a diverse array of hydrophobic ligands, indicating the potential for antigen specific activation and the possibility of self tolerance of these cells. The ability of NKT to produce IL-4 has suggested their ability to drive differentiation of Th2 responses although investigations using NKT deficient ($CD1d^{-/-}$ or $\beta2m^{-/-}$) mice have not supported a role for these cells in such responses (Hong et. al., 1999). However, there is evidence to suggest that NKT cells do play an important function in Th2 responses, Vα14 TCR transgenic mice which have a tenfold increase in NKT cell numbers, possess elevated serum IgE and IL-4 levels (Bendelac et. al., 1996), whilst NKT cell activation *In Vivo* promotes Th2 associated immunity (Burden

et. al., 1999 and Kitamura et. al., 2000). NKT cells have also been shown to be involved in Th1 inhibition through the action of various cytokines, namely IL-4, IL-10 and TGF-β. Sonoda et. al.,(1999) showed NKT cells are essential for controlling anterior chamber-associated immune- deviation (ACAID) which prevents the eye from damage by inflammatory immune responses. Bone marrow (BM) derived NKT cells have also been observed to prevent graft versus host disease following BM transplantation in an IL-4 dependent manner, which highlights the involvement of these cells in the prevention inflammatory immune responses (Zeng et.al., 1999). NKT cells have been shown in some models to mediate cytotoxic responses to tumour lines *In Vitro*, suggesting a role for tumour rejection (Smyth et. al., 2000). *In Vivo* treatment of mice with IL-12 induces tumour rejection which has been suggested to be NKT cell mediated (Dale et. al., 2000) however, alternative studies (Takeda et. al., 2000 and Smyth et. al., 2000) indicate that IL-12 induced tumour rejection may be model and dose dependent. Finally, Takahashi et. al., (2000) have shown that NKT cell subsets stimulated *In Vitro* with αGalCer-pulsed dendritic cells, mediate perforin dependent cytotoxicity against the U937 tumour cell line.

T cell receptors are diverse and specific for multiple antigens. αβ heterodimers associate with four invariant membrane proteins. Three of these are collectively known as the CD3 complex and associates with a third protein identified as the ζ chain. These CD3 complex proteins form the T cell receptor complex with the TCR and transduce signals following interaction of the α-β heterodimer with antigen.

During B and T cell development, sub populations of cells differentiate with restricted B and T cell receptor repertoires which possess high copies of particular clones. This enables responsiveness by reducing the need for clonal expansion and differentiation upon primary infection.

1.7: γδ T cells

This population plays a pivotal role in immune regulation, tumour surveillance and primary responses (Hayday et al., 2003). γδ T cells are derived from thymic precursors which also differentiate into αβ T cells and are positively selected on cognate self antigen. These cells can recognise antigens without the requirement of

processing or presentation (Groh et al., 2002 and Davis et al., 1999). A subset of γδ T cells (Vγ9.Vδ2 T cells) respond to mycobacterial compounds, secrete Th_1 cytokines and proliferate during bacterial infections. These cells represent 1-5% of the circulating T cell population, and it is suggested that their development is determined by the strength of signals received through the T cell receptor (Pennington et al., 2005). These cells have identified several aspects of cellular immunology, non MHC restricted activation of human T cells and T cells possessing limited TCR diversity within certain tissues. γδ T cells can both effect and suppress immune functions, and have been shown to promote B cell production of antibodies in immunodeficient animals, suggesting a mechanism promoting the observed high antibody titres in AIDS patients (Smith et al., 2000).

1.8: Complement soluble mediators

Secreted molecules incorporate more than thirty proteins of the complement cascade which circulate in inactive forms known as zymogens within plasma. Activation of these proteins occurs within tissues and the blood itself, initiating a complex cascade of proteins culminating in opsonisation of target pathogens and formation of the membrane attack complex (MAC) which disrupts the cell membranes of invading organisms. The complement cascade is further discussed in **section 1.17** and details how complement components tag microbial surfaces and initiate their elimination, either via MAC formation or through interaction with receptors on many cells of the immune system.

As the complement system is at a continual 'tick over' state many host cells that come into contact with serum components express complement regulatory proteins in order to prevent 'bystander' attack. The main regulators are CD55, CD46 and CD59 and are further discussed in **section 1.20**.

1.9: Acquired Immunity

Antigen specific responses, not involving pre-evolved recognition of cellular motifs, are mediated by acquired immune mechanisms. Professional Antigen Presenting Cells (APCs) and T cells are equipped to respond to specific peptide antigens presented by major histocompatibility (MHC) molecules. Antigen recognition is a

two phase process requiring activation of naïve T cells via presentation of antigen to the T cell receptor. This is mediated by TCR binding peptide/MHC complexes activating T cells, which then migrate to sites of infection mediated by chemical signals. The second phase of T cell responses is the direct killing of target cells expressing the same peptide antigens in the context of MHC class I. While Dendritic cells are involved in the initial phase, APCs located within the site of infection may be required to mediate CTL mediated killing

1.10: Antigen presenting cells (APC)

Four main categories of antigen presenting cells have been identified which are able to modulate cellular immune responses through antigen presentation pathways. These include monocytes, dendritic cells, B cells and endothelial cells, (Verhsselt., 1997), all of which possess innate receptors for a multitude of cytokines. Stimulation of these receptors results in cell activation and increased expression of MHC molecules, Fc receptors, co-stimulatory molecules such as B7 and CD11, adhesion molecules and further cytokines and enzymes.

1.11: Dendritic cells

DCs are said to be the one of the central players in all immune responses of the innate and adaptive immune system, involved in maintenance of inflammatory conditions and being critical to self tolerance induction and maintenance (Rossi et al., 2005). DCs are referred to as professional antigen presenting cells due to their ability to activate naïve T cells. Immature DCs found in non-lymphoid tissues have been shown to possess a strong ability for antigen capture and processing, but appear to be poor at presentation to naïve T cells. Maturation of DCs has been shown to reverse these characteristics and can be induced by stimulation with many cytokines, IL-1, CD40-L (Brossart et al., 1998), endotoxin (Brossart et al., 1998) and by cross linking CD43 (Corinti et al., 1999). Brossart et al., have identified that while mature DCs can be derived from CD14 progenitor cells when cultured in the presence of GM-CSF and IL-4, GM-CSF may not be the most vital cytokine required as CD40 ligation alone promotes the differentiation and maturation of DCs without GM-CSF or IL-4. Most DCs are classified based upon their location, Langerhans cells within the epidermis and mucous membranes within the heart, kidney and lungs, interdigitating DCs in the T cell areas of secondary lymphoid tissue and thymic medulla and circulating DCs

which constitute 0.1% of the blood leukocytes (Hashimoto et al., 1999). Another population sub set is the plasmacytoid DC (pcDC) which has been observed in secondary lymphoid tissues and in certain malignancies and inflammatory conditions. Austyn (2002) describes the capacity of plasmacytoid DCs to induce regulatory T cells, suppressing immune responses. Austyn states that while this subtype may be involved in the induction of tolerance to self antigens, both in the thymus and periphery, they can contribute to innate defences against viral infection and can also acquire the ability to initiate adaptive immune responses. It also appears that due to pcDCs apparent selective recruitment to certain tumours, their role in cancer immunity is of significant importance. Serial analysis of gene expression (SAGE) and flow cytometry have been used to identify tags from DCs and monocytes and genes involved with MHC molecules and cytoskeletal structure are shown to be up-regulated within DCs (Hashimoto et al., 1999).

The primary function of dendritic cells is to capture antigens within the tissues and present them to T cells within lymphoid organs (Yang et al., 1998) and at the site of infection. They maintain surveillance of their environment by both binding and processing antigens (Fanger et al., 1996). Upon stimulation with innate antigens such as LPS or cytokines, several responses of DCs can be observed, the up-regulation of MHC class II, reduction in Fc receptors and production of IL-12. It is during this process that DCs migrate to T-cell areas of lymphoid organs in order to present signal 1 and 2 to $CD4^+$ and $CD8^+$ T cells (Coyle et al., 2001). Binding of the anaphylatoxins C3b or C4b to antigen enhances APC function in the activation of T cell clones via complement receptors upon APCs (Arvieux et al., 1999).

Langerhans cells are bone marrow (BM) derived and, in contrast to tissue macrophages are not phagocytic and act via the process of bulk endocytosis (Murphy et al., 1986; Yanagihara et al., 1998). These cells play an important role in cutaneous cellular responses and are involved in contact hypersensitivity through interaction of MHC class II. Follicular dendritic cells are not leukocytes and are of stromal origin and interact with B cells within germinal centres (Tew et al., 2001).

It has been recently shown in several studies that DCs and NK cells reciprocally activate one another during immune responses, the original concept of this cross-talk

being identified in 1999 by Fernandez et al., in which the *In Vivo* anti-tumour response of mouse NK cells was enhanced by DCs. DC activation of resting NK cells *In Vitro* often requires direct cell to cell contact, but DC derived cytokines including IL-12, IL-18 and type I interferon are also required for the induction of various NK effector functions (Hamerman et al.,2005, Granucci et al., 2004). Fewer studies have been carried out assessing the nature of NK cell activation of DCs, although Picciolli et al., 2002, showed that NK cells pre-activated with IL-2 are potent activators of DCs, both alone and in the presence of inflammatory stimuli. They also identified that the outcome of NK-DC interaction is tightly regulated by the ratio of the two cell types. Low NK: DC ratios result in DC maturation whereas high NK: DC ratios result in NK mediated killing of DCs. Several possibilities have been raised for the site of interaction between these cell types. Sites of inflammation are suggested, as resident immature DCs can be found and NK cells migrate to these sites in response to inflammatory mediators. Buentke et al., 2002 have shown that NK cells and DCs are in direct contact at the dermal sites of yeast infection. Alternatively, Ferlazzo et al., 2004, showed that human NK cells are found within both inflamed and non-inflamed lymph tissues where they may contact maturing DCs which have migrated from sites of inflammation or infection.

1.12: Monocytes

Activated monocytes and macrophages are involved in both acquired and innate immune responses and direct their responses through several mechanisms including phagocytosis. Monocytes display a variety of activation states similar to Dendritic cells and both cell types possess Fc and complement receptors (C3R) that enable them to phagocytose opsonised cells and antigenic complexes. While playing critical roles in the removal of pathogens, toxins and dying cells, they are also involved in the presentation of antigen to effector T cells. Monocytes however, are unable to stimulate naïve T cells and possess Toll like receptors that bind bacterial components such as mannose and LPS. Monocytes and macrophages are able to generate cytokines such as IL-12, IL-1, IL-10 and TNFα (Kakumu et al., 1997). The combined effects of IL-1 and TNFα on T cells are shown to induce IL-2 receptor expression and IL-10 has a direct effect on B cell activation (Gan et al., 1999). IL-10 is also known

to direct monocytes maturation into macrophages (Allavena et al., 1998) while down regulating cytokines from macrophages (Moore et al., 1994).

1.13: T-cells

There are two predominant T cell subtypes that are involved in the mediation of acquired immunity, namely $CD4^+$ T cells (helper T cells) and $CD8^+$ T cells [cytotoxic T cells (CTLs)]. Internally processed proteins are generated by the proteosomal complex and generated peptides are loaded onto MHC class I, which is present on most normal host cells. MHC class II molecules are expressed by Anitgen Presenting Cells and present processed antigen, obtained from external proteins, which is recognised by the T cell receptor on $CD4^+$ cells. APCs express both class I and II MHC molecules and present antigen to receptors on other cells. T cells are activated when 'signal 1' is generated via the MHC/peptide-T cell receptor interaction, in combination with 'signal 2', which is derived from the interaction between CD80 and CD86 on APCs which are recognised by CD28 on T cells. The combined signals stimulate T cells to secrete a range of cytokines. Post activation, T cells migrate to the site of antigen stimulation, driven by chemotaxis in response to proinflammatory signals released at sites of infection (Banchereau and Steinman, 1998). Helper T cells ($CD4^+$) secrete multiple cytokines which direct and perpetuate immune responses dependent upon danger signals presented. $CD4^+$ mediated help is described as being of either Th_1 or Th_2 in specificity (Infante et al., 1999). Th_1 responses constitute release of cytokines, including IL-12 and IFNγ, which are essential for cell mediated immunity (Behar et al., 1995). Binding of IFNγ onto tumour cells is known to upregulate MHC class I (increasing expression of tumour antigens) and Fas, which binds Fas ligand expressed on T and NK cells, inducing apoptosis of colon carcinomas and hepatocellular carcinoma cells, thereby increasing recognition and potential killing by CTLs (Wang et al., 1997). Th_2 responses involve expression of cytokines such as IL-10, IL-4 and IL-6, which are critical for humoral mediated immunity and the development of antibodies (Infante et al., 1999). $CD8^+$ CTLs mediate the direct killing of infected host cells, expressing peptides from intracellularly processed pathogens, and potentially, tumour cells expressing altered self antigens.

1.14: B-Cells

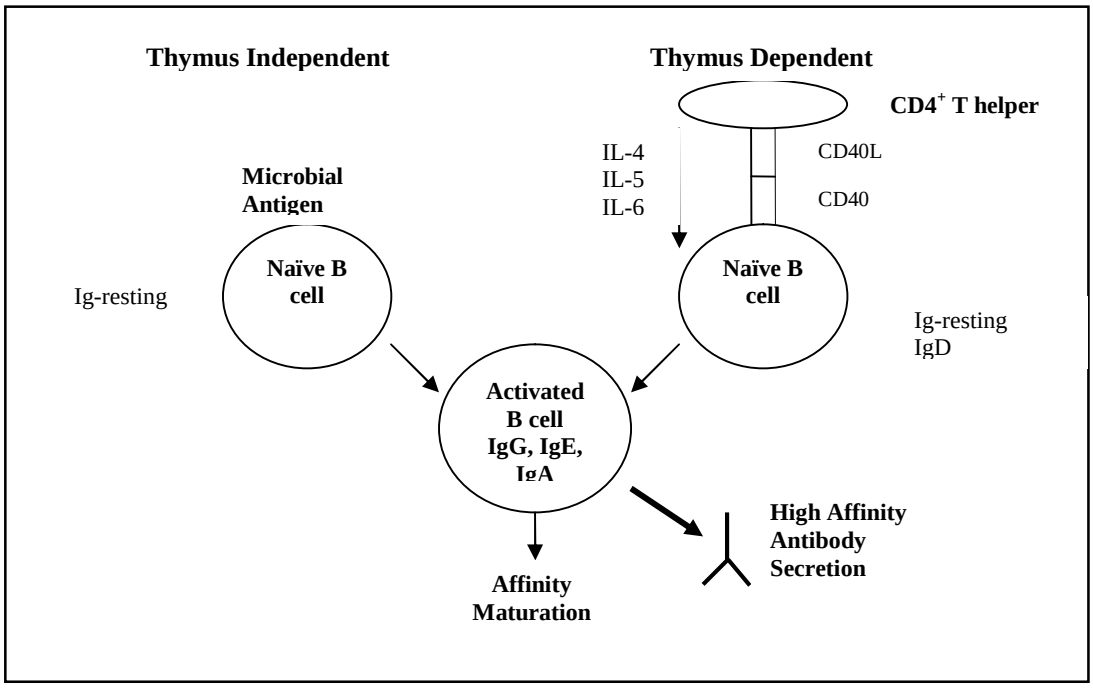
B cells are important mediators of humoral immunity expressing immunoglobulins (Ig) as surface molecules in combination with the secretion of soluble immunoglobulins. These proteins bind antigens, recognising specific peptide sequences with respect to tertiary structure (Gold et al., 2002). Binding of surface Ig to external antigens stimulates internalisation of the complex, enabling processing and presentation of antigenic peptides in the context of MHC class II pathways (Defrance et al., 2002). Thymus independent antigens, such as microbial components, are capable of directly activating B cells, while most antigens are not involved with direct activation of B cells and require accessory signals provided by helper T cells (Thymus dependent antigens). It is recognition of identical antigens by activated helper T cells that bind B cells, through CD40, and signal proliferation. Activated helper T cells produce cytokines such as Il-4, IL-5 and IL-6 that promote differentiation and clonal expansion of B cells into plasma Ig secreting cells (Infante et al., 1999; Gause et al., 2001). Differentiation of B cells into plasma cells restricts their ability to interact with alternate antigens via surface immunoglobulins and also with T cells. B cell interaction with follicular DCs in the germinal centres of lymph nodes induces maturation into memory B cells capable of secreting antigen neutralising antibodies without the requirement for thymus dependent interactions. **Figure 1.4** summarises the maturation of B cells.

1.15: Effector Cells

Co-stimulation of effector cells within the tumour environment is being extensively studied, and evidence suggests that multiple stimulatory pathways are required for CTL recognition and cytotoxic function. Receptor /ligand systems identified include PD-L1/PD-L2 expressed by tissue cells and the respective PD-1 receptor on CTLs, B-cells and myeloid cells (Freeman et al., 2000) and $CD4^+$ T cells (Latchman et al., 2001). More recently, OX40 has been shown to be expressed on activated T cells with ligation of the OX40-Ligand leading to clonal expansion and proliferation of these T cells (Sugamura et al., 2004). Binding of this receptor on $CD4^+$ T cells also increases the expansion of antigen specific T helper cells (Weinberg et al., 1998), and this interaction is also shown to be essential in the generation of memory T cells and their survival mediated by OX40 ligand expression on APCs. OX40 therapy in tumour bearing animals through the use of OX40 antibodies or OX40-ligand

immunoglobin fusion proteins has been shown to enhance anti-tumour immune responses and generated increased tumour free survival in various models (Ali et al., 2004).

Figure 1.4: Summary of B cell Maturation



1.16: Complement

The complement system is a major effector of the humoral /innate branch of the immune system, and components of this early defence mechanism involve a classical and alternative pathway of activation (Matzinger, 1994).

The complement system consists of more than thirty soluble glycoproteins, which are largely synthesized by liver hepatocytes, blood monocytes, tissue macrophages and epithelial cells of the gastrointestinal and genitourinary tracts. The complement components interact in a regulated enzymatic cascade, stimulating products which facilitate antigen clearance and generate an inflammatory response. Both the classical and alternative activation pathways result in production of a macromolecular membrane-attack complex (MAC) that is able to induce lysis of many cells, bacteria and viruses. The whole cascade enables rapid destruction of many invading organisms and mediates the solubilisation and clearance of immune complexes.

1.17: Complement cascade and activation pathways

The two major activation routes for complement include the alternative and classical pathways (Kinoshita, 1991). However, a third pathway known as the lectin route has been described (Reid, 1994). **Figure 1.5** diagrammatically represents an overview of the complement cascade, identifying the three initiating stages and the terminal pathway which leads to the formation of the MAC complex.

The classical pathway is commonly initiated by the formation of soluble antibody complexes bound to particulate antigen or antigenic targets upon whole cells, e.g. bacteria, and involves the 'heat labile' serum components C1, C2, C3 and C4. Antigen/antibody complexes induce conformational changes within the Fc region of the IgG or IgM molecule, exposing a binding site for the C1 component. Upon binding, C1q then C1r undergo conformational changes, activating C1r protease, which cleaves C1s, forming an active enzyme. C1s in turn cleaves C4 and C2 leading to formation of soluble C4a and reactive C4b, which forms a magnesium dependent reversible complex with C2. C4b2a complex is then generated which is the C3/C5 convertase of the classical pathway (CP). It is this convertase which is able to cleave multiple C3 and C5 molecules, greatly enhancing the initial signal.

The Alternative Pathway, unlike the CP, does not require the presence of antibodies, and is initiated by various cell surface constituents, which are foreign to the host. The AP is continuously activated at low levels within plasma, and C3 can undergo spontaneous hydrolysis with Bb to form the initial fluid phase convertase of the alternative pathway. It is the C3b component, which binds to foreign surface antigens, and binds factor B in a Mg^{++} dependent manner. The B region is cleaved by factor D and becomes the actual C3 convertase of the AP, C3bBb. The AP can also be initiated by the CP, as C3bBb cleaves C3 molecules into C3b subunits of new C3 convertases and forms a positive feedback loop. C3b deposition also leads to opsonisation for phagocytosis and generation of C5b, which initiates activation of the terminal pathway.

The lectin pathway for complement activation is similar to the classical pathway, except that it is initiated by mannose binding lectin (MBL) that recognises

carbohydrates containing mannose or N-acetyl glucosamine residues on bacteria and other micro-organisms.

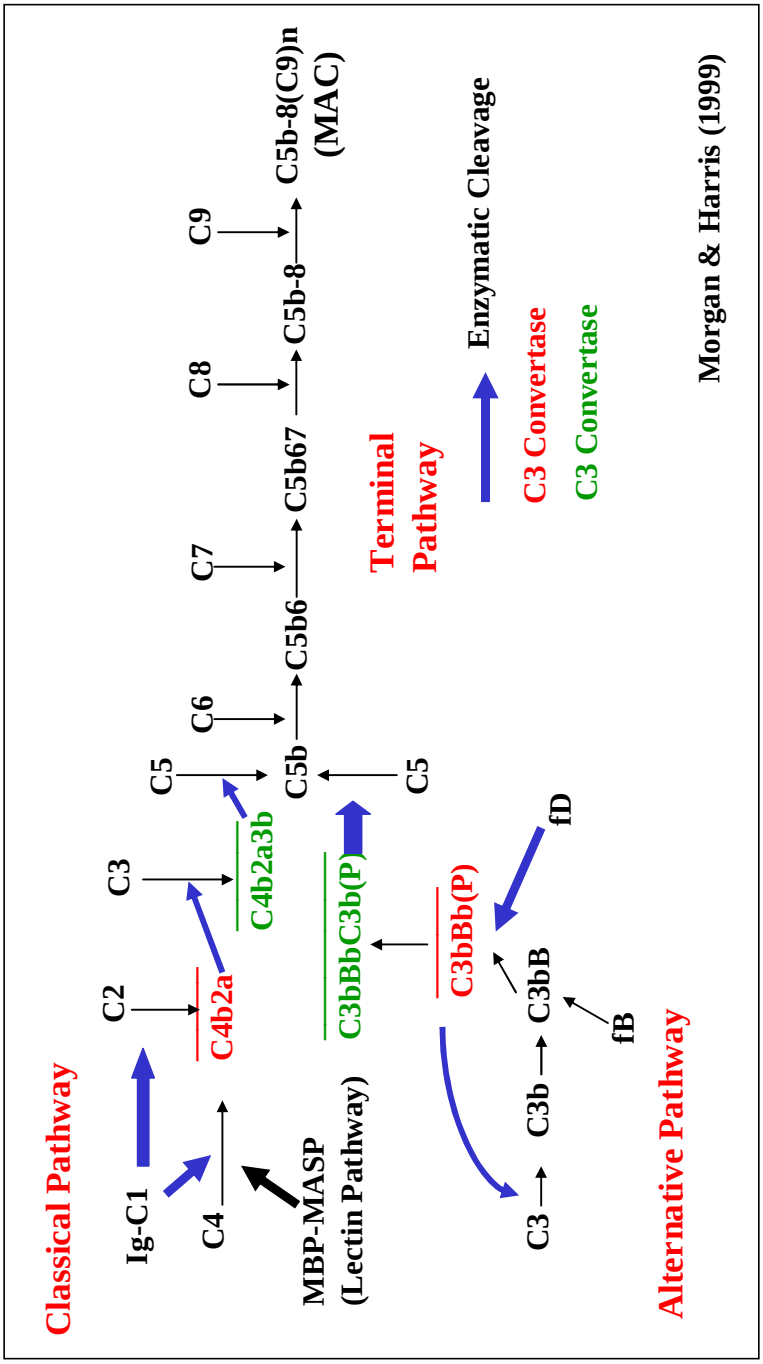
MBL is homologous to C1q and binds to serine proteases to form C1-like complexes. The cell bound structure catalytically cleaves C2 and C4, and progression of the pathway continues like that of the classical pathway.

The Terminal pathway becomes initiated when either the CP or AP C5 convertase cleaves C5 to form C5b. This in turn binds to C6, which is released by polymorphonuclear leukocytes at sites of inflammation, allowing recognition of C7. This C5b-7 complex is hydrophobic in nature and binds to lipid membranes, C8, and multiple C9 molecules. When polymerisation of the C9 structures occurs, penetration of the cell membrane, MAC development and perforation of the target is achieved.

1.18: Anaphylatoxins C3a and C5a

C3a and C5a are products of all three activation pathways and are involved in the generation of inflammation via histamine, leukotrienes and other mediator release from mast cells and basophils. These Anaphylatoxins also induce vasodilation and contraction of smooth muscle leading to increases in vascular permeability, while C3a receptors present on neutrophils, monocytes and eosinophils have direct modulatory effects on B cells. C5a has also been shown to attract phagocytic cells and trigger lysosomal enzyme release. Both C3a and C5a are chemotactic and induce migration of leukocytes to inflammation sites (Till et al., 1986, Frank & Fries, 1991,), as well as inducing aggregation of platelets. Many cell types express receptors to C5a and C3a including hepatocytes that have shown to become activated upon C5a binding stimulating synthesis of acute phase proteins (McCoy et al, 1995). These acute phase reactants promote the inflammatory stimulus mediating the immune response, and include C-reactive protein that is capable of opsonisation, agglutination and activation/enhanced stimulation of the classical pathway. This generation of inflammatory responses help protect tissues by removing infectious agents.

Figure 1.5: Summary of the Complement Cascade



1.19: Complement receptors

Many cells of the immune system possess surface receptors which bind the C3 and C4 fragments which deposit upon the surface of pathogens and initiate cellular responses. **Figure 1.6** summarises the distribution and function of some complement receptor proteins.

Complement receptor 1 (CR1) on the surface of macrophages and neutrophils binds to pathogen bound C3b or C4b and facilitates uptake and phagocytosis. In this respect the components act as opsonins, being the principal function of complement, and act by enhancing phagocytosis initiated by binding of IgG to Fc receptors or by IFNγ stimulation of the effector cell.

Complement receptor 2 (CR2) is expressed by B cells and forms part of its co-receptor complex. The CR2 interaction with iC3b, C3d or C3dg fragment upon the pathogens surface amplifies the signal initiated when the B cell receptor associates with its specific antigen.

Complement receptors 3 and 4 are β-integrins and associate with pathogen bound iC3b. They are expressed by phagocytes and augment Fc receptor and CR1 activation of phagocytosis. However unlike CR1 interaction with C3b, iC3b association with CR3 is sufficient to stimulate phagocytosis alone. These complement receptors also act as cellular adhesion molecules and are involved in leukocyte adhesion to endothelial cells during inflammation.

Figure 1.6: Summary of complement receptor protein distribution and function

Receptor	Ligand	Function	Cell types
CR1	C3b, C4b	Promotes C3b and C4b decay. Stimulates phagocytosis and erythrocyte transport of immune complexes	Erythrocytes, macrophages, B cells and FDCs
CR2	C3d, C3dg, iC3b	Part of B cell co-receptor	B cells and FDCs
CR3	iC3b	Stimulates phagocytosis	Macrophages, monocytes,leukoctes and FDCs
CR4	iC3b	Stimulates phagocytosis	Macrophages, monocytes, polymorphonuclear leukocytes

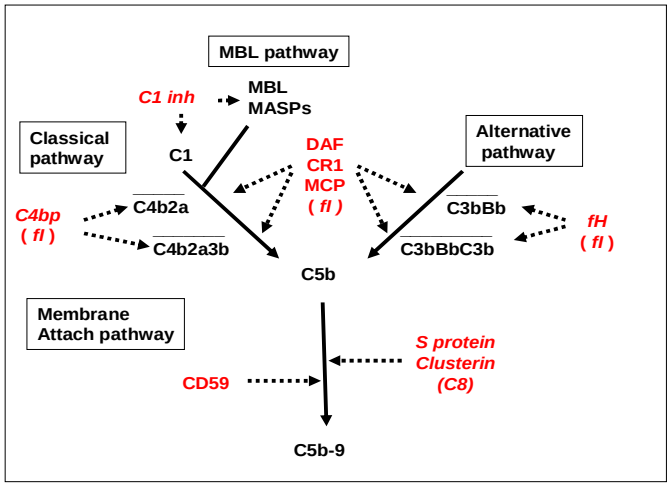
1.20: Complement regulation

Complement is continually active at a slow 'tick over' rate on all cells of the body; the very nature of the pathways allows a small stimulus to generate a large and potentially harmful response in the absence of regulation. Uncontrolled activation would not only produce harmful effects, but would result in the exhaustion of complement

proteins, leaving an individual compromised. It is due to this that the complement pathway is tightly regulated by several mechanisms, including inherent instability of pathway enzymes and MAC precursors and membrane bound regulators of complement, which prevent non-specific 'bystander' attack of autologous cells. The membrane bound regulators of complement activation include complement receptor 1 (CD35), decay accelerating factor (CD55), membrane cofactor protein (CD46) and protectin (CD59). **Figure 1.7** displays a summary of complement regulation.

CD46 (Membrane cofactor protein) contains four Short Consensus Repeat domains, is a glycoprotein containing a transmembrane domain, and acts as a cofactor for factor 1 and promotes the cleavage of C3b to enzymatically inactive iC3B or C4b to C4c and d (Seya et.al. 1989). Factor H is also critical to this reaction as, in its presence, factor 1 is able to cleave C3b. CD46, while not decaying C3 convertase itself, aids CD55 decay accelerating activity and is expressed on all circulating cells, including platelets, T cells and B cells, but not on human erythrocytes. CD46 has been shown to only inactivate C3b which is bound to the same cell, which indicates a requirement for correct orientation of MCP to C3b on the membrane (Seya et. al., 1989).

Figure 1.7: Regulators of the complement cascade



Kojima et. al. (1993) state that CD46 preferentially inactivates alternative pathway convertases and C3b molecules covalently bound to other membrane proteins

including C3b dimers. Seya indicates that in the absence of factor I, CD46 is able to stabilise both pathway convertases leading to increased C3 deposition. Cross-linking of CD46 on activated monocytes is shown to down regulate IL-12 production, which is vital for maintaining a Th1 response (Smith et al, 1997 and Kaminski et al, 1999). Th1 responses drive cell-mediated activity enabling destruction of bacteria and viruses, and interestingly, the measles virus uses CD46 as a ligand to gain entry into monocytes, thus inhibiting IL-12 production (Karp et al, 1996)

Complement receptor 1 is also a transmembrane glycoprotein that possesses decay accelerating activity, and cofactor ability for factor 1 in cleaving cell bound C3b to iC3b and subsequently C3d and fluid phase C3c.

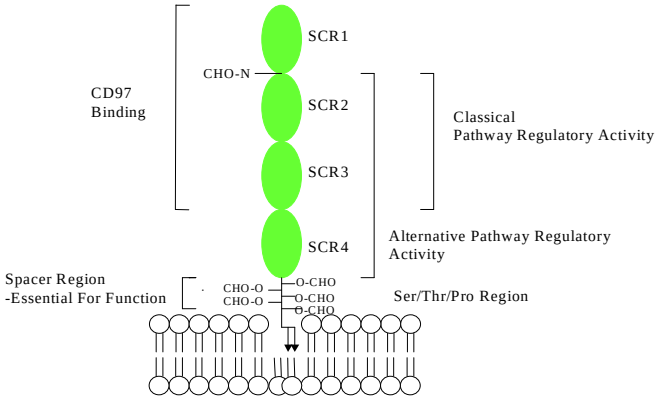
CD59 (Protectin) is widely expressed on most circulating cells including erythrocytes, and functions to inhibit the later stages of the terminal pathway. This is achieved by binding to the C5b-8 complex, allowing binding of one C9 subunit, but preventing subsequent unfolding and membrane insertion, preventing further C9 binding and membrane disruption. CD59 has also been associated with erythrocytes binding to T cells via the adhesion molecule CD2 (Whitlow et al, 1990). This interaction has been suggested to be an important co-stimulatory association for T cell activation (Menu et al, 1994). CD59 has been shown to cluster/associate with other GPI anchored molecules, which contain tyrosine kinases (Stefanova et al, 1991), and has been shown to be present in the form of a homodimer on nucleated cells. This dimerisation is suggested to be important for signalling through CD59 (Hatanaka et al, 1998). Recent work on CD59 expression patterns on breast carcinoma sections has also shown that a loss of CD59 is associated with poor patient prognosis, which contributes to theory that complement regulatory proteins are all associated and regulation is balanced between varying specific functions (Madjd et. al., 2003 and Watson et. al., 2005).

1.21: CD55 structure and function

CD55 or Decay Accelerating Factor is a 70,000 MW membrane protein that is anchored by a C-terminal glycolipid (Medof et.al. 1986) and protects cells from autologous complement deposition. From the amino terminus it consists of four Short Consensus Repeat (SCR) domains, each containing approximately 60 amino acids.

The four SCR domains are followed by a region rich in extensively glycosylated Ser, Thr and Pro residues, which contain sites for the addition of O-linked oligosaccharides. The 17 terminal amino acids are essential for the formation of a glycophosphatidylinositol (GPI) anchor that links the functional protein to the cell membrane. **Figure 1.8** demonstrates the structure of complete CD55. The CD55 gene is located within the complement regulatory locus on the long arm of chromosome 1, band q3.2 (Lublin et.al. 1987); it spans approximately 40kb and is comprised of 11 exons.

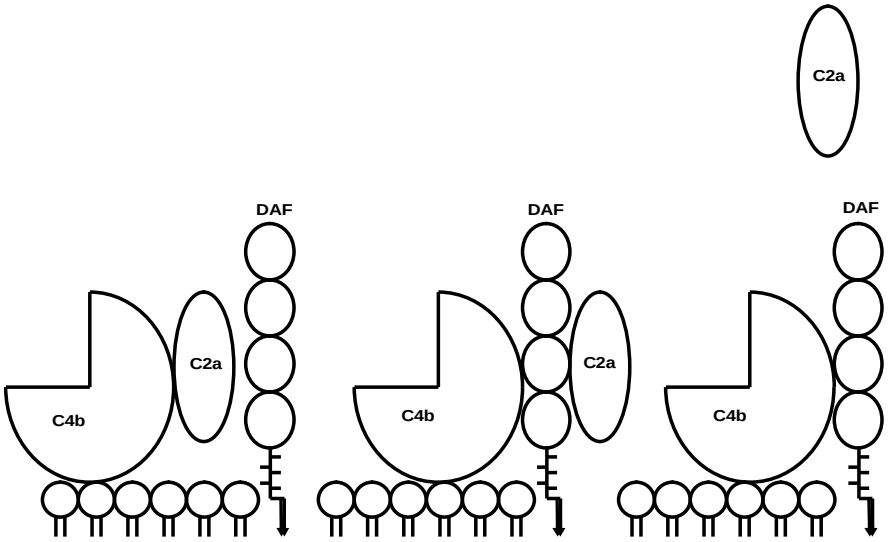
Figure 1.8: Structure of CD55 (DAF)



CD55 functions to accelerate the decay of either C4b2a or C3bBb and corresponding C5 convertases. CD55 acts intrinsically by rapidly dissociating C2a and Bb from C4b and C3b respectively which are bound to the cell membrane to which it is anchored and not on convertases bound to alternate targets, thus preventing assembly of the C3 convertase (Fujita et. al. 1987). CD55 regulatory activity is displayed in **Figure 1.9**.

Evidence has shown that CD55 activity is predominantly upon preformed convertase complexes containing Bb or C2a and that binding of either fB or C2 to C3b or C5 respectively (Fujita et al., 1987). Binding affinity analysis of CD55 and its complement associated ligands has identified significant differences between interactions with single subunits and complete complexes (**Figure 1.10**).

Figure 1.9: CD55 inhibition of complement activation pathways (C4b2a catalytic C3 convertase of the classical activation pathway)



CD55 has a low affinity interaction with both C3b and C4b on the cell surface enabling their release from the regulator post convertase decay, allowing 'recycling' of CD55, enabling further interactions with newly formed convertases (Morgan & Harris 1999).

Figure 1.10: Summary of convertase subunits and apparent association

	Unit	Apparent association constant (appKa)
Alternative C3 Convertase	C3b	$45nM^{-1}$
	Bb	$67\ nM^{-1}$
	C3Bb	$910\ nM^{-1}$
Classical C3 Convertase	C4b	$0.45\ nM^{-1}$
	C4b2a	$530\ nM^{-1}$

The difference between association constants clearly identifies that for the alternative pathway convertase there is an increase of 15 to 20 times for the complex and that for the classical pathway convertase of more than 1000 times (Pangburn et al.,1986). Pangburns's research assessed CD55's ability to accelerate release of radio labelled Bb from C3bBb bound to zymosan in the presence of various inhibitors of complement. The SCR domains of CD55 are commonly found within C3b/4b binding proteins and can be cross-linked to either subunit on the cell surface, indicating that the inhibitor is in close contact with these fragments (Kinoshita et al.,

1986, and Reid et al., 1986). The apparent low affinity association of the subunits is likely vital for CD55 effective activity, enabling dissociation from the smaller units post decay of the intact complex, allowing further interaction with other convertase structures.

Decay accelerating activity is located in SCR domains 2 and 3 for the classical pathway and domains 2 to 4 for the alternative pathways (Brodbeck et. al. 1996). Disruption of hydrophobic C^{147} and F^{148} residues within the SCR 3 domain have shown to produce significant inhibition of DAF regulatory activity within both the Alternate and Classical Pathways. Although SCR 1 is usually stated to show no involvement in CD55's complement regulatory function, antibody blocking studies have shown that CD55 SCR 1-2 hybrid transfectants possess the ability to regulate AP activity (Christiansen et. al. 2000).

X-ray crystallography has identified that critical contacts are formed with a hydrophobic region located between SCR domains 1 and 2, and that mutation of residues in previous analysis may have simply altered the structure of this hydrophobic patch.

The nature of some of the critical residues has also been supported by a molecular model for factor H, based on NMR data. Factor H shares both structural and functional homology to CD55 and this model predicted that convertase interaction sites may include a positively charged region extending from SCR 2 into a groove at the SCR2/3 interface, R^{100}, R^{101}, $KKK^{125\text{-}127}$, and with a hydrophobic pocket within SCR3, V^{121}, F^{123}, L^{147}, F^{148} and L^{171} (Kuttner-Kondo., 1996).

The primary function of SCR 1,2 and 3 appear to be mediating CD97 binding, which is a leukocyte antigen belonging to the family of seven trans-membrane spanning proteins (Hamann et. al. 1998). It has been suggested that CD97 is involved in the initiation of inflammatory responses, and its structure indicates signal transducing capacity, implying that CD55 is involved in alternative functions other than C regulation (Gray et. al.1996). The Ser/Thr/Pro region of CD55, while having no direct convertase regulatory activity, acts as an essential spacer region, projecting SCR domains from the plasma membrane, enabling function (Coyne et. al. 1992).

CD55 deficiency is associated with PNH (Paroxysmal Nocturnal Haemoglobinuria), which is characterised by sensitivity of erythrocytes to autologous complement mediated lysis. Leukocytes and platelets are also affected, as they are generated from haematopoietic stem cells which lack the ability to add GPI anchors to the CD55 protein (Kinoshita et. al. 1985).

The 'Inab' phenotype has been described in individuals who exhibit total loss of CD55 expression (Telen et. al. 1993). However, these individuals do not suffer from erythrocyte haemolysis, indicating the role CD59 has in protecting these cells from complement attack.

1.22: CD55 expression and distribution

Decay accelerating factor is widely expressed on most circulating cells including erythrocytes, leukocytes, NK cells and endothelial cells. DAF has also been located in the urinary, gastrointestinal and exocrine systems (Medof et. al. 1987 and Cosio et. al. 1989), placenta, spermatozoa, eyes, and is associated with the sub endothelial matrix (Hindmarsh et. al. 1998). Soluble forms have also been identified in several biological fluids including synovial fluid, CSF and urine (Cosio et. al. 1989).

Various tumour lines have been shown to over-express CD55 including colorectal, gastric, ovarian and osteosarcoma cells (Spendlove et. al. 1999). These altered expression levels can be up to 100 fold greater than normal cells (Li et.al. 2001), and while this over-expression conveys resistance to complement mediated attack (Koretz et. al. 1992), it is therefore potentially a cofactor for tumour development. Strong CD55 expression has also been observed in stromal tissue (Niehans et. al. 1996), and it is suggested that this may be due to shedding from tumour cells, a theory supported by the presence of soluble CD55 within serum and all forms being capable of inhibiting complement activity. CD55 presence within the extracellular matrix (ECM) is shown to be up regulated on tumours in response to increased production of vascular endothelial growth factor (VEGF). Morgan et. al. (2002) showed that VEGF stimulates the release of matrix degrading metalloproteinases, specifically MMP-7, which in turn releases intact CD55 from the ECM. As elevated CD55 levels are often much greater than needed for complement regulation, this would imply that decay

accelerating factor possesses alternative functions other than C regulation (Carrington et. al. 2001).

1.23: CD55 as a signal transducer

GPI anchored molecules are often involved in cell signalling and DAF has been shown to associate with other membrane proteins and trigger cell activation. The GPI anchor of CD55 has been shown to be vital for signalling, as when it is replaced with the transmembrane and cytoplasmic domains of membrane cofactor protein, all signalling is subsequently lost (Shenoy-Scaria et. al. 1992). Although the complete signalling pathway for decay accelerating factor is unknown, it is understood that DAF associates with *src* family tyrosine kinases in microdomains within the membrane, called lipid rafts or detergent-insoluble glycolipid enriched domains (DIGs). Upregulation of glucose consumption and phagocytosis has been noted when CD55 is stimulated by monoclonal antibodies on phagocytes (Shibuya et. al. 1992). CD97 has also been shown to act as ligand for SCR 1 to 3 of CD55 and is thought to transduce signals from CD55 to cytoplasmic molecules (Fujita et. al. 1987). In 1988, Davis et. al. showed that a mitogenic signal could be transduced within T lymphocytes by costimulating CD55 following sub-mitogenic stimulation with PHA, resulting in T cell proliferation. Phosphatidylinositol phospholipase C treatment of T cells, cleaving the GPI Anchor of CD55, abrogated this costimulation indicating that T lymphocytes are stimulated via this mechanism. Spendlove et al. (2006), have shown that soluble, recombinant CD55 inhibits T cell activation and proliferation, an effect that can be blocked by the addition of CD55 (SCR 1-3) specific antibodies. Current research continues to examine the signalling events involved with this interaction.

1.24: Complement as a target for immunotherapy

While complement is a vital mechanism of the immune system, as a key mediator of inflammation, it is also a major causative agent in host tissue destruction. Both clinical and experimental evidence has identified complement activity in several inflammatory diseases and recently in neuro-degenerative disorders such as Alzheimer's disease and Multiple Sclerosis (Kirschfink et. al. 2001).

The complement system plays a pivotal role in the rapid destructive rejection of xeno-transplanted organs, a process call Hyperacute Rejection (HAR). Two main

approaches for complement inhibition have been suggested: the blockade of interactions between native xenoreactive antibodies and xenograft endothelium; targeting the α galactosyl epitope [major xenoantigen (Rother and Squinto 1996)]; and blockade of complement activation with either soluble inhibitors or transgenic models expressing human regulators. Leventhal et. al. (1994) showed that cobra venom factor prolonged transplant survival by depleting C3a and C5a levels. However, by generating and increasing convertase activity, increased endothelial damage was also observed. Soluble complement regulators have been shown to restrict the action of complement within body fluids at multiple sites of the cascade reaction, as when C1 inhibitor is combined with heparin, inhibition of classical pathway activity occurs (Dalmasso and Platt 1993). Protection of exogenous cells has also been achieved by transfection with the cDNA of human membrane cofactor protein (CD46), decay accelerating factor (CD55) and protectin (CD49). Heckl-Ostreicher et. al. (1996) produced porcine endothelial cells expressing human CD59 that, both with and without the addition of soluble regulators, inhibited complement mediated cell lysis. Xenoperfused human DAF and CD59 transgenic pig hearts and kidneys have been produced expressing analogous and elevated levels of complement regulators compared to normal human epithelium, capable of inhibiting complement activation and producing a reduction of morphological alterations, which are usually indicative of HAR.

1.25: Cancer: Tumour development and immunity

Cancer has become the major cause of mortality in the developed world, with 1 in 3 people in the UK alone being diagnosed at some point in their lifetime, with 25% of all deaths being cancer related (Cancer Research UK, data compiled for 2002). Tumour cells are derived from many normal host cells with the tumour extracellular matrix providing structural support and being a rich source of vital components for maintaining growth.

Tumours develop through the successive development of genetic variations. This instability enables tumour cells to potentially mutate at an increased rate, providing responsiveness to varying environmental stresses, and out grow normal host cells. Genetic instability is often identified in tumour development and is derived from the

degree of heterogeneity within individual tumours and also between tumours of the same type (Shih et al., 2001). Microsatellite instability is shown to enable nucleotide mutation rates which are up to three orders of magnitude greater than in cells possessing normal mis-match repair genes (Bielas et. al., 2006). Solid tumours have also been shown to display chromosomal instability, although most tumour cells appear to develop displaying either chromosomal or nucleotide instability (Cahill et al., 1999). Tumours can develop from the accumulation of many somatic mutations within a single cell, often originating from a single spontaneous mutation during the normal growth and development cycle (Calvert et al., 2002). Three common gene types are known to incorporate these mutations, namely DNA repair genes (p53-cell cycle), oncogenes (ATM-repairs DNA breakages) and tumour suppressor genes (BRCA1- regulate cell growth), (Nowak et al., 2002).

Initial dogma suggested that tumour cells were invisible to the immune system due to the thymic deletion of T cells recognising self antigens. Several studies opposed this argument and have shown that immune responses are directed towards tumour targets. CTLs have been cloned from patients bearing regressing tumours, demonstrating that T lymphocytes are able to recognise and lyse tumour cells (Boon et al,.1997 and Rosenberg 1999). Identified tumour associated antigens so recognised have been classified into six main categories including, viral antigens [HPV (Human Papilloma Virus); EBV (Epstein Barr Virus)], Cancer Testis (CT) antigens (MAGE, NY-ESO-1), over-expressed self antigen (Her2/neu, P53), fusion proteins (bcr-abl), mutated antigens (MUM-1,p53, CDF-4) and differentiation antigens (MelanA/MART-1, tyrosinase, gp100). Sahin et al., (1995) demonstrated that high titres of IgG antibodies could be isolated from tumour patients with specificity to a range of antigens, which were also recognised by $CD8^+$ T cells and are displayed in the cancer immunome/SEREX database. IFNγ receptor or signal transducer and activator of transcription-1 (STAT-1) knockout mice showed the presence of increased susceptibility to spontaneous and carcinogen induced tumour development. IFNγ was identified as enhancing cell mediated immunity in conjunction with having direct cytotoxic effects upon tumour cells. Following these observations $RAG2^{-/-}$ mice, deficient in all T, natural killer and B cells were identified as being significantly more susceptible to spontaneous and carcinogen induced tumour formation (Shankaran et.

al., 2001). This group also showed that formed tumours were more immunogenic than tumours developing in immunocompetent mice (Dunn et al., 2002).

Matzinger (1994), states that the immune system responds to danger signals as opposed to foreign antigens. It appears that prevention of autoimmune disease, recognition of self antigens, is controlled by several pathways acting in synergy. Within the thymus, auto reactive T cells are selectively deleted and regulatory T cells are developed. Regulatory T cells can also be stimulated within the periphery in order to promote anergic responses to self antigens. Naïve T cells bind to cognate peptide-MHC complexes and if this signal is combined with co-stimulatory signals, T cell activation is induced. Therefore, in cases of cellular damage, self reactive T cells may become activated due to the combined signals of danger (co-stimulatory molecules on APCs) combined with antigen recognition. Once damage is repaired, APCs would no longer deliver co-stimulatory signals and remaining T cells encountering self antigen will be either anergised or driven to become regulatory T cells.

The principles of immunosurveillance and immunoediting are reviewed by Dunn et al (2004) and (2002). The principles described suggest that the immune system actively sculpts tumour cells and that there are three main stages leading to tumour formation. The elimination phase describes how early tumour cells form and express distinct tumour specific markers, generating proinflammatory signals that initiate molecules of both the innate and adaptive immune system, which results in the eradication of mutated/altered self cells. The equilibrium phase states that some cells may survive this initial attack and are 'edited' by the immune system, promoting loss of antigens or stimulatory signals generating new populations of tumour variants. The third phase is identified by the 'escape' of tumour variants that are no longer detected by immune effectors.

During tumour development, healthy cells are present which do not provide danger signals to the immune system which is said to be immunologically ignorant (Zinkernagel 2002). As the tumour develops, some cells become necrotic and release intracellular components and 'danger signals'. Necrotic cells also release many cytokines in order to stimulate new blood vessel formation. VEGF, an angiogenic cytokine is released and also inhibits activation of APCs (Gabrilovitch et al., 1996),

which may prevent immune recognition/responses and may possibly promote regulatory T cell formation. Many solid tumours secrete cytokines such as transforming growth factor β (TGFβ) and IL-10 in order to promote protection from tissue damaging effects such as delayed type hypersensitivity responses. Cytokines may not only inhibit immune responses but may switch responses to Th_2 responses preventing CTL mediated attack (Heriot et al., 2000). It appears that selective pressure exerted by the immune system, while preventing development of many potential tumour cells, actually sculpts cell variants to develop mechanisms of escape, which may ultimately lead to tumour formation. Therefore it is vital to continue assessment of up-regulated tumour antigens in order to understand methods of immune evasion and to identify potential antigenic targets that may be exploited.

1.26: Development of anti-cancer immunotherapeutics

The discovery that tumours can be recognised by the immune system has lead to extensive characterisation of molecular mechanisms underlying recognition. Many tumour associated antigens have been identified and have enabled the development of several immunological approaches in the development of cancer vaccines. Strategies include exploitation of molecularly defined T cell epitopes, antibody based strategies, cytokine therapies, immune modulators, DNA vaccines, whole cell vaccination strategies, hormone based therapies and combinations of all of these methods. Therapeutic vaccines are required to stimulate both cellular (Cytotoxic T cells and T helper cells) and humoral responses, generating antibodies. Prophylactic vaccines for cancer present numerous obstacles in that tumour cells may present a variety of potential antigens. The administration of vaccines should be tailored to individuals and difficulties arise when predicting who will generate specific tumours. However, the advent of genomic screening for cancer and identification of predisposition could enable the prophylactic treatment of high risk patients.

Main line treatment of cancer still involves surgery to remove the tumour and/or the administration of secondary treatments such as radio/chemotherapy and drugs/chemicals aimed at targeting cancer cells. The development of immunological strategies, augmenting immune responses and overcoming regulatory mechanisms, offers greater treatment specificity than many current strategies. The main classes of

immunotherapy in phase II and III clinical trials are adjuvants, antigens and immunostimulants, cell therapies and monoclonal antibodies as reviewed by Durrant et al., (2003).

Cytokines and adjuvants are generally used to augment existing immune responses to specific antigenic targets and a limited number have been approved for treatment of cancer, including IL-2 for the treatment of melanomas and certain blood cancers. An array of monoclonal antibodies has been developed and are being utilised in clinical settings as mentioned in **section 1.27**. Many antibodies function by binding target antigens, coating target cells and recruiting immune effector mechanisms for destruction of the tumour cells. Several antibodies also act by signalling events within tumour cells, inhibiting growth signals, or by preventing the binding of cytokines to their respective receptors.

Immune stimulants are utilised to promote immune responses and in many cases to promote a Th_1 phenotype, enhancing cellular responses with particular focus on cytotoxic T cells. Several immune stimulants target the activity of antigen presenting cells, thus augmenting cytokine responses that promote T cell activation. An example of an immune stimulant is Histamine dihydrochloride (Ceplene^{TM}), which has been shown to enhance cytokine mediators in stimulating T cells (Hamill et al., 2003 and Schmidt et al., 2002). Clinical trials have assessed this compound, alone and in combination with IL-2, for the treatment of metastatic melanoma (phase III). QS-21 (Stimulon^{TM}) is a saponin extracted from the bark of *quillaja saponaria* and has been used as adjuvant in phase III melanoma trials (Sondak et al., 2002). It promotes both cellular and humoral responses when mixed with soluble antigens and is shown to stimulate antibodies to GM2 when administered with GM2-keyhole limpet haemocyanin (Chapman et al., 2000).

As previously mentioned, cytokines are produced at sites of inflammation to stimulate and regulate many immune effectors. Actimmune® ($IFN\gamma_{1b}$) has been used to promote the activity of effector T cells and has been launched in the U.S. for use with severe malignant osteoporosis. In early phase I and II clinical trials, it has been used with some success in the treatment of ovarian cancer (Durrant et al., 2003).

Cellular vaccines use tumour cells or cell lysates obtained from cancer bearing patients. These combinations of mixed tumour antigens have the potential to stimulate both cellular and antibody mediated immune responses. However, many responses observed appear to be weak, potentially due to the non pathogenic nature of the antigens given as opposed to viral or bacterial antigens. Canavaxin™ is a polyvalent melanoma vaccine comprising of three cell lines expressing over 20 characterised antigens. The abundance of antigens presented in a single depot is thought to promote both $CD4^+$ and $CD8^+$ cellular immunity, which is boosted by the presence of BCG acting as an immune stimulant. Analysis has shown that responding melanoma patients demonstrate cellular delayed type hypersensitivity responses to certain antigens, which suggests a correlation between immune response with clinical outcome (Hsueh et al., 2002).

Dendritic cell vaccines are viewed as potentially successful therapies as DCs are required to stimulate naïve T cell responses. Two main approaches are currently used in clinical settings: DCs are isolated from patients which are then pulsed with tumour antigens and re-administered back to the patients; Heat Shock Proteins, which chaperone MHC binding peptides from patients' tumours, are isolated and injected back into the patients. They bind to receptors on dendritic cells, enabling processing and presentation of tumour antigens (Basu et al., 2001). HSPPC-96 (Oncophage®) has been used within phase III melanoma trials and patients were shown to generate an increase in melanoma specific T cell activity, with 5 out of 23 patients showing long term survival (Assikis et al., 2003).

Synthetic vaccines incorporate synthesised molecules that are used to stimulate specific antibody mediated or cellular responses. GMK is a vaccine to ganglioside (GM2) which over-expressed in many cancer types such as melanoma, breast and prostate. Synthetic GM2 is conjugated to KLH, which acts as a carrier protein, providing T helper cell responses, promoting maturation of humoral responses from IgM to IgG antibodies. The adjuvant QS-21 is also added to the treatment and early phase I/II trials patients were shown to developGM2 specific antibodies that killed tumour cells (Chapman et al., 2000).

Anti idiotype vaccines are also used in clinical settings, utilising antibodies that mimic tumour antigens. These antibodies stimulate host responses to the immunogen and thus drive the development of CTLs which also recognise the tumour antigen. Mitumomab (Bec2) is an example of an anti idiotype that has been used in early trials showing increased survival rates of patients with small cell lung cancers (Chapman et al., 2003). BEC2 is an investigational anti-idiotypic monoclonal antibody that is designed to prevent or delay the recurrence of certain types of tumours. The BEC2 antibody mimics the ganglioside GD3 which is a tumour associated antigen. By mimicking this antigen, BEC2 stimulates a stronger immune response to cells expressing natural GD3. In limited pilot studies, preliminary findings suggest that BEC2 has the potential to stimulate the body's immune system to identify and eliminate residual tumour cells, preventing the recurrence of tumours, and prolonging survival in patients with limited disease small cell lung carcinoma. The company in cooperation with its partner Merck KGaA is currently evaluating BEC2 in an international Phase III clinical trial for this indication.

1.27: Antibodies as therapeutic agents

The first indication that monoclonal antibodies could be used as therapeutic agents was published in 1982, showing that a ‘tailor made’ mouse anti-idiotype antibody was developed and used on one patient with B cell lymphoma. The subject showed a complete response to this brief treatment indicated by a remission period for 17 years (Miller et. al. 1982). This finding sparked a new field of research aiming to develop the ‘magic bullet’ for the treatment of many diseases including cancer. However early animal and human trials failed to reproduce the efficacy of the initial anti-idiotype study. Commonly observed problems included the high levels of toxicity produced by antibody conjugates and the development of HAMA (Human Anti Mouse Antibody) responses which prevent the efficacy of repeated antibody therapy. An exception to this is the success of the mouse monoclonal antibody orthoclone OKT3 which was approved by the FDA for organ graft rejection in1986.

During the last decade, development in molecular and recombinant DNA technology has enabled significant advancement in the production of high affinity, non-immunogenic, highly efficacious monoclonal antibodies. One of the most significant

criteria for antibodies which are to be used in clinical applications is the ability for repeat high dosing of subjects without producing high toxicity levels. Several strategies have been developed to overcome the HAMA response:- Human/ Mouse chimeric molecules containing human constant sequences with mouse variable domains, humanised antibodies where the only murine domain is that of the Complementarity Determining Regions (CDRs) and completely human antibodies produced via phage libraries and transgenic mice (Vaughan et. al. 1998). All these modifications allow a reduction in immunogenicity, although chimeric, humanised and complete human antibodies may still induce antibody responses to variable regions, although this has been shown to occur in less than 12 percent of immune competent cases (Clark M, 2000). Molecular modification also increases the ability of antibodies to recruit host effector responses either via the complement system or through the activity of cytotoxic cells, which is achieved by including human IgG constant regions, a potent ability for the treatment of malignancy. Humanisation of murine immunoglobulins has improved their effectiveness in immunotherapy, as half lives can be increased from approximately 20 hours (rodent) to more than 21 days. The presence of the human Fc region enables binding to endothelial FcR_n (Brambell) receptors which salvage molecules and enable avoidance of intra-cellular breakdown. Addition of human Fc regions also reduces the development of human anti-mouse antibody (HAMA) responses, and enhances capacity for complement recruitment and induction of antibody dependent cell cytotoxicity responses.

There are several possible modes of action to be considered in antibody development in order to generate a specific and effective therapy. There are three main modalities, those which act by blocking or modulating responses such as through the inhibition of growth signals within target cells, targeting specific antigens thereby opsonising the cellular targets and inducing effectors, or by inducing signal transduction pathways through cell receptors in order to generate a specific response (stimulation of apoptosis or co stimulation of immune effectors turning weak responses into effective anti tumour activity). Conjugate therapy has also shown to be an effective treatment of malignancies by combining antibodies with cytotoxics, such as chemotherapeutic agents or radio isotopes, delivering the active agent directly to the tumour site.

Many monoclonal antibodies are undergoing trials and several have been approved by the FDA for treatment of a multitude of conditions. **Figure 1.11** shows the current status of several therapeutic antibodies. Many of the monoclonal antibodies currently used are for the treatment of malignancy and utilise different modalities to induce their anti tumour effects.

Figure 1.11: Summary of current FDA approved antibodies

Year FDA approved	Product	Target	Indication
1986	Muromonab-CD3 (OKT-3)	CD-3	Transplant rejection
1994	Abciximab (ReoPro)	GP11a/111b	Percutaneous transluminal coronary angioplasty
1997	Rituximab (Rituxan)	CD20	B cell lymphoma
1997	Dacliximab (Zenapax)	IL-2r	Transplant rejecton
1998	Basiliximab (Simulect)	IL-2r	Transplant rejection
1998	Infliximab (Remicade)	TNF	Crohn's disease, Rheumatoid arthritis
1998	Palivizumab (Synagis)	RSV Respiritory Syncytial Virus	RSV in infants
1998	Trastuzumab (Herceptin)	HER-2/neu	Breast cancer lymphomas, prostate cancer
2000	Mylotarg (Gemtuzumab Ozogamicin, CMA-676)	CD33	Acute Myelogenous Leukemia
2002	Ibritumomab tituxetanytium^{90} (Zevalin,IDEC-Y2B8)	CD-20	Non-Hodgkin's Lymphoma
2002	Alemtuzumab (Campath, Campath-1H)	CD-52	B cell chronic lymphocytic leukaemia
2003	Tositumomab I^{131} (Bexxar)	CD20	Low grade Non-Hodgkin's Lymphoma
Pending (Germany 1995)	Edrecolomab (Panorex)	Anti idiotype antibody for protein 17-1A (Ep-CAM)	Colorectal cancer
2005	Epratuzumab (LymphoCide) Fast track product designation post Phase II trials	CD-22	B cell malignancies Treatment of moderate to severe SLE
2004	Cetuximab (IMC-C225) Erbitux (Imclone)	Epidermal growth factor	Colorectal,pancreatic & Head and Neck

		receptor (EGFR)	cancers
Phase III trial 2004	Mitumomab (IMC-Bec-2)	Anti idiotype against ganglioside GD3	Small cell lung cancer
2003 Phase II/III trial indicated no increased survival	ABX-CBL (Abgenix) New Focus ABX-EGF-Panitumumab (fully human MoAb)	CD 147 EGFr	Steroid resistant graft Vs host disease (GVHD) Lung, breast and colorectal cancer

Rituximab (Rituxan) is a chimeric human/murine monoclonal IgG1 specific for the CD20 antigen present on normal and malignant mature B lymphocytes. 80 percent of all Non-Hodgkin's Lymphomas are B cell malignancies and >90% express CD20, which is not found on hematopoietic stem cells (White.C, et. al. 2001). Rituximab has shown therapeutic efficacy in triggering the tyrosine phosphorylation of intracellular proteins, specifically activation of protein kinase C and upregulation of *Myc* (Cragg et.al. 1999). Several studies have shown that cross linking of the B cell receptor with antibody stimulates both growth arrest and apoptosis in both normal and malignant cells (Glennie et.al. 2000). As Rituximab possesses human gamma 1 heavy chain and kappa light chain constant regions, it is effective in mediating ADCC and CDC in the presence of human complement, and within mature B cells it also sensitises chemoresistant cells to toxins and chemotherapy agents (White et.al. 2001). Rituximab was given FDA approval in 1997 for use in refractory low grade or follicular lymphoma that express CD20 antigens. An interesting factor is the role in which complement regulatory proteins have been shown to affect the outcome of rituximab therapy. While rituximab is known to generate both CDC and ADCC responses, Golay et al (2000) showed that complement mediated lysis varied from 100% lysis to complete resistance on several Burkitt's lymphoma cell lines. They also showed that by blocking CD55 with specific antibodies, CDC responses were increased, indicating that heterogeneity of rituximab responsiveness is in part, affected by complement regulatory proteins. Cerny et al (2002) also confirmed how blocking of both CD55 and CD59 can overcome rituximab resistance and potentially sensitise target tumour cells to apoptosis and complement mediated lysis. These novel findings promoted Bannerji et al (2003) to determine whether CD55 expression

could be utilised to determine rituximab therapy outcome, however findings indicted CD55 and CD59 levels did not predict clinical responsiveness to rituximab therapy in chronic lymphocytic leukaemia patients. Di Gaetano et al, (2003) demonstrate that complement activation is fundamental for rituximab therapeutic activity *in vivo* by using syngeneic knockout mice lacking C1q. This also promotes the use of complement regulatory protein inhibitors for combined potential therapeutics.

Trastuzumab (Herceptin) is a humanised IgG1 monoclonal antibody derived from Chinese hamster ovaries specifically targeting human epidermal growth factor receptor 2. This receptor signals via the Ras pathway to increase cyclin D1 expression, which is correlated with reduced sensitivity to hormonal and endocrine therapy. Trastuzumab therapy alone has produced responses in 15 percent of patients with metastatic breast cancer (Johnson, 2001), and a randomised trial has indicated that in combination with chemotherapy, median survival is increased by 25 percent (Slamon D, 2001). Trastuzumab was given FDA approval for single and combination therapy for breast cancer overexpressing HER2 (c-erbB-2) in patients that have shown limited responses to chemotherapy alone.

Gemtuzumab Ozogamicin (Mylotarg) is a recombinant humanised antibody conjugated with the cytotoxic anti-tumour antibiotic calicheamicin (Wyeth Laboratories, PA). It binds CD33 antigen, which is an adhesion protein, expressed on the surfaces of leukaemic blasts and immature normal cells of the myeloid lineage. CD33 is not found on the surface of normal haematopoietic stem cells. Upon binding to its antigen, myelotarg is internalised and releases the calicheamicin derivative into the cells, which binds to DNA, resulting in DNA double strand breaks and cell death. Adverse reactions noted in clinical trials have included bone marrow suppression with median recovery of neutropenia of approximately 40 days, related fevers and less commonly hepatotoxicity. Myelotarg is approved in the treatment of patients with CD33 positive acute myeloid leukaemia in first relapse.

CD52 is an antigen expressed on normal and malignant lymphocytes, macrophages, eosinophils and monocytes (Nemecek et al, 2002). Alemtuzumab (Campath-IH) is a humanised rat IgG1 antibody reactive with CD52 and is approved for the treatment of B cell chronic lymphocytic leukaemia (Berlex laboratories, Richmond, CA). This antibody is thought to produce an anti leukaemic effect via the activation of

complement and antibody-dependent cytotoxic effector mechanisms. However, its efficacy has been shown to be transient if not followed by other therapies such as haematopoietic stem cell transplantation (Nemecek et al, 2002).

Tositumomab I^{131} (Bexxar) is a murine monoclonal IgG targeting the CD20 antigen on B-lymphocytes, which is labelled with $Iodine^{131}$. It has been used in the treatment of low-grade Non-Hodgkin's lymphoma and has two modes of effect. By targeting the CD20 antigen, apoptosis is induced and the antibody is also able to mediate antibody dependent cellular cytotoxicity. Tositumomab also acts by delivering ionising radiation to the tumour site. Kaminski et. al. (1996) showed that a non myeloblative dose regime in 34 patients with NHL gave a 79 percent overall response rate and only 14 percent of cases developed HAMA responses. Tositumomab was given FDA approval in 2003 for the treatment of patients with CD20 positive, follicular, non-Hodgkin's lymphoma (NHL), with and without transformation, whose disease is refractory to Rituximab.

Edrecolomab (Panorex) is a murine IgG2a monoclonal antibody directed against the surface glycoprotein 17-1 antigen/ epithelial cell adhesion molecule (Ep-CAM). It is shown in cases of colorectal carcinoma that following surgical resection of primary tumours, outgrowth of distant metastasis remains difficult to prevent. However, adjuvant monoclonal antibody therapy may provide an effective method to prevent/ reduce the spread of tumour cells. Antibodies such as Panorex activate the classical pathway of complement activation, which can result in direct lysis of tumour cells or act by the recruitment of leukocytes to the tumour site. However, in clinical trials 52% of patients still show recurrence of disease after 7 years, which has been associated with the enhanced expression of complement regulatory proteins shown on tumours (Gelderman et al, 2002).

Epratuzumab (Amgen) is an anti CD22, humanised monoclonal antibody which is being assessed for the potential treatment of Lymphoma, non-Hodgkin's Lymphoma (NHL) and certain auto-immune conditions. Epratuzumab has been used in conjunction with rituximab treatment in relapsed and refractory NHL and generated a beneficial antilymphoma effect without showing an increase in toxicity. Amgen have also developed conjugated forms of epratuzumab with the radioisotope ^{90}Y which is

being used in a phase III clinical trial for the potent treatment of aggressive lymphoma (Juweid, 2003). In June 2003 epratuzumab received orphan drug status in the United States as a treatment for NHL.

ERBITUX™ (Cetuximab) is an IgG1 antibody which targets and blocks Epidermal Growth Factor Receptor (EGFR), which is expressed on the surface of cancer cells in multiple tumour types. Erbitux binds EGFR and prevents interaction of natural ligands with their receptor, preventing phosphorylation of signalling pathways. It has been analysed in second line colorectal cancer, lung, pancreatic, ovarian, head and neck cancers. Adverse affects include rash, diarrhoea, nausea abdominal pain and vomiting.

ABX-CBL IgM murine antibody which targets CD147 and initiates killing via complement mediated lysis. Human CD147/neurothelin or EMMPRIN is a member of the immunoglobulin super-family and is weakly expressed on human leukocytes, granulocytes and red blood cells. ABX-CBL depletes activated T and B cells as well as resting and activated monocytes and dendritic cells *in vitro*, whereas resting lymphocytes remain unaffected (Joachim Deeg et al, 2001). It is has been assessed in patients with steroid-refractory graft versus host disease, generating 44% survival rates 6 months following treatment (Joachim et al, 2001).

Immune conjugates have thus been assessed and some success has been achieved, as is the case for tositumomab. However, immunotoxins have shown limited success, as several trials have shown the emergence of dose limiting toxicities such as vascular leak syndrome, hypersensitivity reactions and CNS toxicity (White e. al. 2001).

ADEPT (antibody directed enzyme prodrug therapy) utilises antibodies conjugated to prodrugs which are directed to the tumour site and activated by local enzymes. This methodology is at an early stage in development and several clinical trials are being carried out utilising prodrugs with short half lives, in order to reduce the necessity for antibody clearance to reduce the level of toxicity generated (Francis et. al. 2002).

1.28: DNA Immunisation

Common methodology for antibody development is based upon cDNA sequence data, from which peptides can be synthesised and utilised for immunisation protocols. However, such strategies are often inefficient, particularly when antibodies are required to recognise a native protein for use in conformational assays such as flow cytometry or in therapeutic applications. Over the last decade, research into genetic or DNA immunisation has enabled many previous problems to be overcome. In 1990, Wolff et al., showed that mice immunised with a naked DNA plasmid showed expression of reporter proteins within muscle cells at the site of immunisation. This technology was then shown to stimulate specific immune responses (Tang et al, 1992), and over the following years, stimulation of both humoral and cellular responses have been widely reported (Donnelly et al., 1997; Tighe et al., 1998). This technology platform has been used to generate antibodies through DNA immunisation, with cDNA sequences encoding specific proteins enabling the animal's immune system to respond to the foreign protein. Although the quantity of protein produced *In Vivo* following DNA immunisation is shown to be low, within the picogram to nanogram range, efficient immune responses are achieved. This is because the foreign protein is expressed directly in or is quickly taken up by professional antigen presenting cells (Takashima and Morita, 1999). Relatively small numbers of activated APCs have been shown following *In Vitro* immunisation to be sufficient to stimulate T cells (Casares et al., 1997). Enhancement of genetic immunisation has also been shown to be effective due to the presence of CpG-motifs found within the plasmid backbone of the DNA itself, activating both dendritic cells (Jakob et al., 1998) and B cells (Krieg et al 1995). Generation of high affinity antibodies has been shown to be achievable through DNA immunisation in many cases, in particular by Kilpatrick et al., whom in 1998 showed that generation of mature IgG subclasses could be obtained. Single chain Fv antibodies have also been obtained from single chain libraries generated from genetically immunised mice (Chowdhury et al., 1998). The benefits of DNA immunisation over many previously conventional protocols are numerous. A concern with purified proteins is that there is always the potential for the presence of contaminating proteins, compared to genetic immunisations which drive a response to only the expressed desired target. A major factor is that protein targets are produced *In Vivo*, incorporating correct folding and enabling post-translational modifications to occur, maintaining native protein

conformation. This being advantageous compared to purified proteins which may become degraded or never obtain native conformation. There is also potential that DNA immunisations, due to low levels of protein being produced, may generate high affinity antibodies, as indicated by Boyle et al. (1997) when generating antibodies to ovalbumin. They showed that although total IgG levels were similar between genetic immunisation compared to standard protein immunisation protocol, avidity of anti-sera generated from the DNA immunisations were found to be 100-1000 times greater.

Berzofsky et al. (2001) review methods for designing and optimising vaccines, and show that DNA vaccines can be extremely efficient at priming immune responses. As mentioned, specific protein can be expressed *in vivo* for processing and presentation by class II MHC, direct transfection of DCs also enables peptide generation and presentation by class I MHC for the driving of CTL mediated responses. Many DNA vaccines have been developed for the treatment of HIV (Wang et al., 1998) and Malaria (Hanke, Schneider et al, 1998), incorporating CTL epitopes into expressed proteins. This same strategy can be used in the treatment of cancer, incorporating CTL epitopes of tumour antigens for the generation of combined cellular and humoral immunity. Several vectors including viral and bacterial have been developed in order to generate effective anti tumour responses by transfection of autologous and allogenic cell lines for cell based vaccines and also for direct delivery of tumour antigens to antigen presenting cells. An example of a viral vector is the DISC-HSV (disabled infectious single cycle herpes simplex virus), which is only capable of a single round of infection, preventing further infection of other cells. Deepak Assudani et al. (2006) state the immunotherapeutic potential of this vector encoding GM-CSF in murine carcinoma models, which resulted in regression of tumours in 70% of mice assessed.

1.29: Anti-tumour immunotherapeutics targeting CD55 as potential therapy

Mutations in SCR 3 of CD55 have been shown to produce the most disruptive effects on its complement regulatory activity, when compared to mutations in other domains (Coyne et. al.1992). An array of antibodies to specific domains within decay accelerating factor (CD55) were tested for their ability to block its regulatory activity, and only two antibodies, specific to SCR 3, completely abolished its function.

Blok et. al. (1997), showed that a bispecific antibody recognising CD55 and tumour cell antigen G250 induced C3 deposition and tumour cell lysis, and that opsonised cells became sensitised to complement mediated lysis. Similarly Zhong et. al. (1995) showed that increased tumour killing could be achieved by CD55 blockade with an antibody to SCR3 of CD55.

Enhancement of CTL epitopes has been used to convert subdominant epitopes into more efficacious sequences, increasing vaccine potency by improving peptide affinity for MHC class I molecules, thereby enhancing peptide competition for MHC complexes on antigen presenting cells. This strategy has been assessed in several cancer models in order to generate increased antigen specific $CD8^+$ responses (Parkhurst et al., 1996, Rosenberg et al., 1998 and Irvine et al., 1999).

Combining immunotherapeutic strategies targeting CD55 could potentially produce a complete anti-tumour response recruiting multiple arms of the immune system. This total effective treatment would incorporate complement recruitment to the site of tumours via antibody dependent cellular cytotoxicity; neutralisation of CD55 complement regulatory activity upon tumour cells and within tumour stroma through antibody mediated inhibition; prevention of T cell inhibition caused by interaction with tumour CD55 via antibody blockade and recruitment of cell mediated cytotoxicity through immunisation with target-tumour associated antigens.

1.30: Heteroclitic epitopes induce tumour immunity

Cytotoxic T cells (CTLs) play a dominant role in the rejection of tumours, mediated through recognition of target antigens presented by self MHC encoded class I molecules. Binding of T cell receptors to their cognate peptide/MHC complex on tumour cells initiates target cell lysis by the CTL, resulting in tumour elimination. In order for CTL precursors to become activated two signals are required: a stimulatory signal which is transmitted via the T cell receptor-CD3 complex, and a co-stimulatory signal which is delivered by professional antigen presenting cells. Within the thymus, a strong signal 1 can induce negative selection of immature T cells regardless of signal 2 (Nossal et. al., 1994). In the periphery, the same strong signal 1 can initiate immunity or anergy, dependent upon the presence of signal 2. Differentiated/effector T cells, in contrast to T cell precursors, are capable of mediating target cell lysis

driven by only a weak signal 1 without the presence of signal 2 (Sette et. al., 1994). Therefore a group of antigenic peptides exist which, while not capable of directly priming immune responses, may serve as targets of activated effector T cells.

Many tumour antigens have escaped immune recognition either by generating a strong signal 1, promoting deletion of self reactive T cells or generating self reactive regulatory T cells, or by being of low affinity and thus poor immunogens. Therefore, the use of low affinity tumour epitopes as immunogens is counter productive in the generation of strong anti-epitope/tumour CTL responses. A direct correlation between antigen immunogenicity and MHC binding affinity/stability of MHC/peptide complexes for class I epitopes has been demonstrated by several groups (Sette et. al., 1994 and Van der Burg et. al., 1996). Dyall et. al. (1998) summarise that if low affinity epitopes from within tumour antigens can be modified, these potentially immunogenic variants could prime CTL responses that are cross reactive with original target peptide sequences. This would exploit a repertoire that may exist specific to the primary sequence which may not be activated due to low affinity, but which may be available for activation by more immunogenic/high affinity variants. In 1979, Solinger et. al. assessed variants of a cytochrome C peptide, and they identified peptides of higher biological potency than the initial peptides as heteroclitic. Crystal structure analysis (Madden et. al., 1995, Garcia et. al., 1996 and Garboczi et. al., 1996) identified that of the 8 to 10 amino acids of peptides binding to MHC class I, half are exposed to the T cell receptor, with the remaining residues being buried within the class I molecule. Dyall et. al. (1998) summarise that 'heterocliticity' has been achieved by amino acid substitution of bases contacting either the class I molecule, the T cell receptor or substitutions of both, all improving the affinity of interaction between peptides and their target molecules.

Numerous studies have utilised heteroclitic epitopes for immunisation against tumours, promoting responses to weak immunogenic self proteins. Dyall et. al. (1998) utilised heteroclitic peptides from a tyrosinase expressed by melanoma cells, and successfully induced CTL mediated regression and moderate protection from tumours in C57/bl mice. Tourdot et. al. (2000) demonstrate the potential for this strategy, assessing thirty two peptides derived from viral and tumour antigens which have undergone residue modification. Modified peptides were shown to possess an

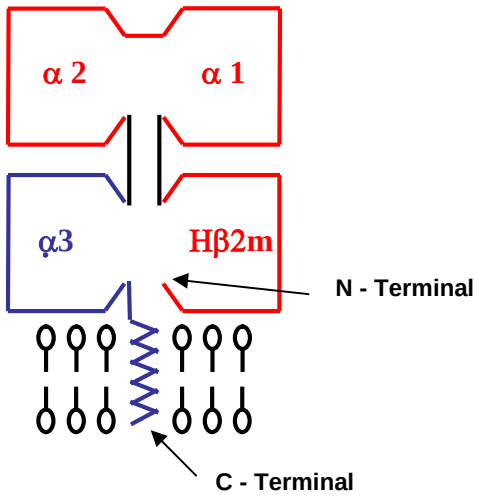
increased affinity for MHC class I, and *In Vivo* analysis identified that enhanced sequences stimulated peptide specific CTL responses which were also specific to naturally processed native epitopes. Graff-Dubois et. al. (2002) analysed the potential for a heteroclitic peptide to be used as a candidate for broad spectrum tumour vaccination. A MAGE-A antigen was identified from multiple tumour types and a heteroclitic peptide was produced. This peptide induced CTLs in HHD HLA-A*0201 transgenic mice, recognising HLA-A2 low affinity peptides derived from MAGE-A1, -A2, -A3, -A4, -A6, -A10 and –A12. These effector cells responded to endogenous antigens in an HLA-A2 restricted manner and recognised human HLA-$A2^+$ MAGE-A^+ tumour cells of various histological origin. Gold et. al. (2003) produced a DNA plasmid construct expressing a variant of the human melanoma antigen gp100. The construct contained a heteroclitic epitope displaying a higher affinity for MHC, which induced protective tumour immunity in melanoma bearing H-2^b mice when used as a vaccine. Ramage et. al. (2004), generated a DNA vaccine encoding human Tie-2, which is a tumour associated antigen expressed on endothelial cells of tumour vasculature. Anchor modifications were carried out to enhance epitope affinity for HLA-A*201, with the most successful constructs driving CTL mediated responses specific to not only both the immunising peptide and the native protein, but also to HLA-A*0201 endothelial cells expressing Tie-2. These results and others, confirm the relevance of heteroclitic epitope immunisation for the stimulation of antigen specific tumour vaccines.

1.31: HHD transgenic mice as a suitable model for DNA vaccine assessment

This project aims to identify epitopes that could be presented by the common human class I antigen HLA-*201. Several strains of class I transgenic mice which still express murine H-2 class I molecules have been derived. However, several previous studies, as summarised by Pascalo et. al. (1997), identified that these mice often preferentially develop H-2 restricted $CD8^+$ responses. Firat et. al. (1999) suggest that unless the third domain of the human molecule is substituted with a mouse domain, the CTL repertoire is inefficiently mobilised from the periphery due to poor interaction with mouse $CD8^+$ molecules. To overcome the observed bias, the HHD model was developed in which the H-$2D^b$ and mouse β2 microglobulin genes were

disrupted and a chimeric human α1, α2 and mouse α3 HLA-A2.1 heavy chain covalently linked to the human β2 light chain was incorporated (**Figure 1.12**).

Figure 1.12: Schematic representation of HHD II, HLA-A2.1 monochains



Adapted from Pascalo et. al. (1997). Red area = Human Origin, Blue area = Mouse Origin.

Several groups have assessed this mouse model, demonstrating that HHD mice can be utilised as a versatile model for pre-clinical evaluation of HLA-A2.1 restricted human tumour associated CTL epitopes. Firat et. al. (1999) assessed a melanoma-based poly-epitope viral construct and demonstrated that CTL responses were observed, specific to five HLA-A2.1 restricted epitopes within single animals. They conclude, through direct comparison, that the size of HLA-A2.1 educated $CD8^+$ peripheral T cell repertoire is larger in HHD than in $A2A2K^b$ transgenic mice. Ramage et. al (2004) observed a greater frequency of antigen specific CTLs in HHD mice, compared to $A2A2K^b$ transgenic mice, immunised with a DNA vaccine expressing an HLA-A2.1 restricted Tie-2 self antigen. They concluded that the HHD model is useful for studying $CD8^+$ responses to over expressed self antigens, while acknowledging that direct comparison with the human system should incorporate the potential for discrepancies between human and mouse T cell repertoires.

1.32: Study Outline

The aims of this project were to generate potential immunotherapeutics targeting the tumour associated antigen CD55. Several methodologies were incorporated in order to develop CD55 neutralising antibodies, and a DNA vaccine with the potential to stimulate both cellular and humoral responses against tumour cells expressing this target molecule.

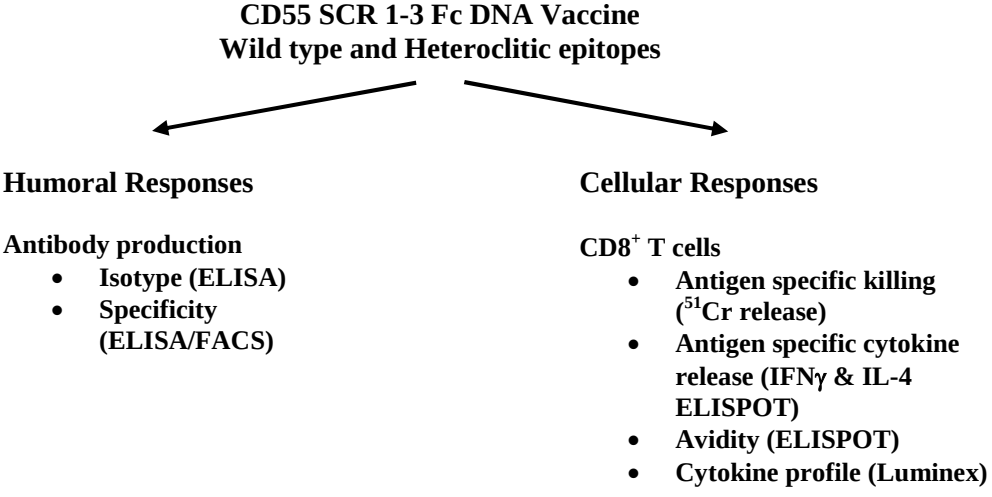
- **Develop monoclonal antibodies specific to active domains of human CD55 capable of targeting tumour cells and neutralising CD55 decay accelerating activity.**

  Raise antibodies through combination of peptide and whole cell vaccination strategies. Assessment to be carried out through combination of ELISA, FACS and complement deposition assays.

- **Develop DNA vaccine capable of eliciting both humoral and cellular responses to human CD55.**

  Generate a DNA construct expressing wild type CD55 linked to human IgG Fc tail for stimulation of therapeutic antibodies capable of targeting tumour cells expressing CD55.

  Incorporation of heteroclitic epitopes into DNA construct for stimulation of HLA-A*201 restricted $CD8^+$ cytotoxic T cells capable of mediating lysis of CD55 bearing tumour cells.



Chapter 2: Materials and Methods

All procedures were carried out using aseptic technique where appropriate and all safety regulations were followed. Standard protocols were either developed or followed for all assays and, where necessary, modifications are incorporated into the protocols for use in specific conditions. All animal work was carried out under a Home Office approved project licence.

2.1 Immunisation Strategies

2.1.1 Peptide prediction and synthesis for immunisation of BALB/c mice

The peptide sequence for SCR 3 domain of human CD55 was obtained from SWISSPROT software (www.expasy.org), accession number P90174. The sequence was entered into the ProtScale program to generate a hydrophobicity plot identifying three potential antigenic regions predicted to be located at the surface of the protein. The peptides were synthesised by John Keyte at the BSAU, Nottingham University. The three peptides were coupled to the carriers KLH (Keyhole Limpet Haemocyanin) and ovalbumin via their N-terminal cystein group using the heterobifunctional cross linking reagent sulpho-MBS (Perbio Science, Cheshire,UK). The carrier protein is dissolved to a concentration of 10mg/ml in PBS and a 25mg/ml MBS is dissolved in dimethylformamide. A 0.1 volume of MBS is added drop-wise to the carrier protein, avoiding high local concentration, and was mixed at room temperature for 30minutes. The activated carrier is then separated from free MBS by filtration on a sephadex G25 or PD10 column (Amersham Pharmacia Biotech, Sweden) in PBS. Target peptide is then dissolved in PBS and added to the carrier in a ratio of 1 Mole of peptide to 50 amino acids of carrier. The final pH is adjusted to 7-7.5 and the mixture is stirred for three hours at room temperature.

The peptide-KLH conjugates were dialysed for 4 hours at 4°C against PBS using 'slide-a-lyzer' cassettes (Pierce/QB Perbio. Rockford. IL.USA). The peptides were emulsified with either Complete or Incomplete Freund's adjuvant (Sigma, Dorset, UK), prior to immunisation as **table 3.1**.

2.1.2 Heteroclitic epitope prediction

In the context of this study, heteroclitic epitopes are defined as class I MHC restricted CTL epitopes whose MHC binding residues can be modified to increase binding affinity without altering CTL receptor recognition.

As per **section 2.1.1** the complete protein sequence of human CD55 accession number P90174 [SWISSPROT software (www.expasy.org)] was obtained and used within several on-line epitope prediction algorithms.

"BIMAS" developed by Parker et al. (1994), access via:
http://www.bimas.dcrt.nih.gov/cgibin/molbio/ken_parker_combofrom

"SYFPEITHI" developed by H.G. Rammensee et al. (1999), access via:
http://syfpeithi.bmi-heidelberg.com

"HLA Ligand/Motif Database" under the direction of William Hildebrand and funded by the National Institute of Health. *University of Oklahoma Health Sciences Center*.

"MHCPEP" – database for MHC ligands and peptide motifs. Rammensee et al. (1999), access via:
http://www.uni-tuebingen.de/uni/kxi/

2.1.3 Peptide immunisation and Sera collection from BALB/c mice

Female BALB/c mice used were aged between 6 and 8 weeks (Charles River, UK) and cared for by the staff at the Biomedical Services Unit at the University of Nottingham.

Immunisations were carried out at two weekly intervals in a maximum volume of 100µl (sample diluted in PBS) using a 1 ml insulin syringe (BD Bioscience, Spain). Immunogen and adjuvant were combined in 1:1 ratio and administered intravenously, sub-cutaneously and intra-peritoneally. Serum was collected via tail bleed extraction, centrifuged at 13,000 rpm for 5 minutes to remove blood cells and serum was stored at -20°C. For whole cell immunisation, target cells were harvested and washed in sterile Phosphate Buffered Saline (PBS). Cells were resuspended in PBS, 10^5 cells per 50µl and combined in a 1:1 ratio with incomplete Freund's adjuvant. 100µl of immunogen were administered intra peritoneally as per protocol (**Table 3.1**).

2.1.4 HHD II transgenic mice

Mice from the HHD II transgenic mouse colony at the animal unit at Nottingham Trent University were used as HLA-A2 mouse models for DNA immunisations. Male and female HLA-A*201/k^b (derived by Nicholas Holmes, Department of Pathology, Cambridge University, Cambridge, UK) between six and twelve weeks of age were used.

2.1.5 Preparation of DNA micro-carriers (gold bullets)

100µl of 0.05M spermidine was combined with 8.3mg of 1 micron gold particles (BioRad) and mixed in a sonicating water bath (Sonomatic, Jencons Scientific Ltd, Leighton Buzzard, UK). 18µg of plasmid DNA at a concentration of 1mg/ml was added to the mixture and sonicated prior to the addition of 100µl of 1M $Cacl_2$ (drop wise). The DNA/gold particle mixture was allowed to stand at room temperature for 10 minutes and then centrifuged at 13,000rpm for 1 minute. The gold pellet was then resuspended in 100% ethanol (Sigma Aldrich). The sample was sonicated and the wash procedure repeated a further two times prior to re-suspension in 1ml of 0.025mg/lm polyvinylpyrrolidone (PVP) (BioRad) diluted in ethanol.

1.5m of tefzel tubing (BioRad) was loaded into the DNA preparation station (BioRad) and dried by passing nitrogen through the tubing for 15 minutes. The DNA coated micro-carriers were loaded onto the tefzel tubing and left to bind for 15 minutes prior to withdrawal of the ethanol. The tubing was rotated 180° prior to continuous rotation for 5 minutes, after 30 seconds nitrogen was passed through the tubing to dry the gold particles. The tubing was removed from the station and 0.5 inch sections of tubing, containing the DNA/gold micro carriers were cut and stored at 4°C with desiccant overnight for use in immunisation the following day.

2.1.6 Mouse immunisation with Helios^{TM} Gene Gun

The Abdomens of mice to be immunised were shaved of fur and the spacer of the gene gun was placed gently onto the skin. A single shot of DNA coated gold micro-carriers, using 400psi pressure of helium was administered.

2.1.7 Epitope-peptide preparation for *In Vitro* Analysis

Peptides synthesised by Alta Bioscience, Birmingham, UK, were suspended in 100µl of sterile DMSO (Dimethyl Sulphoxide) (Sigma, Poole, UK) at a concentration of 40mg/ml. Peptides were then combined with sterile PBS at a 1:1 ratio to produce peptide in solution at 20mg/ml concentration.

2.1.8 MHC stabilisation assay with T2 ($TAP^{-/-}$) cell line

T2 lymphoblastoid cells are deficient in the intracellular peptide transporter TAP (transporters associated with antigen processing), which is used to translocate peptides, that are generated within the cell cytosol by the protesome, to the rough endoplasmic reticulum for assembly within the major histocompatibility complex. As a consequence of peptide binding, the class I MHC molecule displays increased stability and dissociates from both calnexin and the TAP protein. In deficient cells, MHC is not stabilised with internal peptide and upon presentation at the surface membrane it is recycled back within the cell. T2 cells can be used to externally load peptide into the binding site of MHC class I molecules which in turn stabilises this molecule at the cell surface. T2 cells are also HLA-A2 restricted and can be used to assess the binding affinity of synthesised peptides, which were predicted to bind, and thus stabilise MHC class I molecules. This project aimed to identify epitopes with moderate to high affinity for MHC class I and the T2 stabilisation assay was used as a viable assessment of predicted binding.

T2 cells are sub cultured and re-fed one day prior to stabilisation assay in order to maintain high viability in this live assay. T2 cells are harvested, pooled and washed via centrifugation at 12000 rpm in serum free media. Cells were washed twice and the cell pellet was resuspended in complete media and cells were counted with trypan blue staining. Cells were diluted to a concentration of 1 x 10^6/ml and 100µl aliquots were seeded in a 96 U-well tissue culture plate. All samples are assessed in triplicate and controls wells set up without stimulus and with two concentrations of DMSO for compensation of results. 50µl of peptides were added at concentrations of 400µg/ml and 100µg/ml along with 50µl of 500ng/ml β_2 microglobulin to each well. The total assay volume was 200µl of complete media. Plates were incubated for at least 16 hours at 37°C with 5% CO_2. 50µl of 2.5mg/ml of brefeldin A (Sigma) was added per well and plates incubated for a further hour at 37°C. Cells were then transferred to a

96 U-well flexi plate that was centrifuged at 1000 rpm for 4 minutes and supernatant blotted from the wells. Cells were then resuspended in 50µl of PBS and this wash step was repeated a further two times. Cells were analysed by indirect labelling of cells with 50µl of 1:100 BB7.2 antibody (HLA-A2 specific) for 30 minutes on ice. Cells were washed as before in PBS two times and 50µl of 1:100 goat anti mouse FITC conjugated $(Fab)_2$ antibodies were added per well and plates were incubated on ice for a further 30 minutes. Cells were washed in PBS three times prior to re-suspension in 200µl of PBS. Labelled cells were then analysed by flow cytometry.

2.2 Eukaryotic cell culture and modification

2.2.1 Eukaryotic cell culture (Storage, defrosting & maintenance)

All cell culture was carried out using aseptic technique in a class II safety cabinet.

791T : **Human ostesarcoma (in house)**
RPMI 1640 (Sigma)
2mM L-Glutamine (Sigma)
10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)

Colo 205 : **ECACC # 87061208**
Human Caucasian colon adenocarcinoma producing carcinoembryonic antigen
RPMI 1640 (Sigma)
2mM L-Glutamine (Sigma)
10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)

CHO : **ECACC # 85050302**
Chinese Hamster Ovary subclone displaying epithelial morphology
Dulbecco's Modified Eagle's Medium F12 HAM (Sigma)
2mM L-Glutamine (Sigma)
10% H.I. Foetal Bovine Serum (Sigma)

CHO DAF Hi : **CHO cells transfected with CD55 Apex construct**
Dulbecco's Modified Eagle's Medium F12 HAM (Sigma)
2mM L-Glutamine (Sigma)
10% H.I. Foetal Bovine Serum (Sigma)
10μg/ml Puromycin (Sigma)

NSO : **ECACC # 85110503**
Mouse (BALB/c) Lymphoblastoid Myeloma
RPMI 1640 (Sigma)
2mM L-Glutamine (Sigma)
10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)

Hybridoma : **Mouse NSO and splenocyte fusion**
RPMI 1640 (Sigma)
2mM L-Glutamine (Sigma)
10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)
1 x HAT Supplement (1 vial 50 x HAT/ 500ml) (Sigma)

NIH 3T3 : **ECACC # 86052701**
Mouse (BALB/c)
DMEM (Sigma)
2mM L-Glutamine (Sigma)
10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)

EL4 : **ECACC # 85121301**
Mouse C57BL/6N ascites lymphoma lyphoblast
DMEM (Sigma)
2mM L-Glutamine (Sigma)
10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)

Murine : **Murine cells harvested from immunised and naive**

Splenocytes		**mice**
		RPMI 1640 (Sigma)
		2mM L-Glutamine (Sigma)
		10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)
		5ml strptomycin/penicillin solution (Sigma)
		0.05mM β-mercaptoethanol
		10mM Hepes buffer (Sigma)
T2	**:**	**Lymphoblastoid**
		RPMI 1640 (Sigma)
		2mM L-Glutamine (Sigma)
		10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)
CHO SF	**:**	**ECACC # 00102307**
		Chinese Hamster Ovary cell line adapted for growth without protein
		CD CHO media (Gibco, Invitrogen,US)
		1 x HT Supplement
		2MM L-Glutamine
PT67	:	**Packaging cell line Obtained from Clontech (California, USA)**
		NIH/3T3 based cell line expressing 10A1 viral envelope protein
		DMEM (Sigma)
		2mM L-Glutamine (Sigma)
		10% Heat Inactivated (H.I.) Foetal Bovine Serum (Sigma)
		Penicillin (100U/ml) and streptomycin (100µg/ml)

All stock cells were obtained from liquid nitrogen storage. The cells were submerged in a 37°C water bath to ensure complete thawing. Cells were transferred to 25ml universal tubes and 1 ml of complete media, cell specific, was added drop-wise to the cells under agitation. A further 5ml of complete media was added slowly to the tubes

whilst gentle agitation was applied. The volume was then increased with complete media to 25ml and the tubes were centrifuged at 1200 rpm for 5 minutes at room temperature. The supernatant was aspirated from the cell pellets which were resuspended in complete media. All cells were then washed with complete media and finally resuspended in media and transferred to T25 tissue culture flasks (Corning). All cells were cultured at 37°C in 5% CO_2 and were maintained by regular replacement of complete culture media and splitting to maintain log phase growth.

If large cell numbers were required, cells were transferred to T175 culture flasks.

Cells were cultured until approximately 80% confluency was reached, at which point they were split in order to maintain healthy cultures. Supernatant was aspirated from adherent cell lines and replaced with 10ml 1 x Trypsin/EDTA (Sigma) and incubated for 10 minutes at 37°C. Non- adherent lines were transferred to 50ml tubes and centrifuged at 1200 rpm for 5 minutes. Trypsinised cells were transferred to 25 ml universal tubes and complete media was added up to 25ml total volume (Serum contained in the media inactivates trypsin activity). Tubes were centrifuged at 1200 rpm for 5 minutes at room temperature and all supernatants were aspirated from the pellets. Cells were resuspended in 10 ml of complete media and 1ml was transferred to a new flask and the cells were maintained

Cells which were to be used in experimental procedures are counted using a haemocytometer with trypan blue (Sigma), staining for viability assessment and used as stated. Some cells were frozen in order to maintain stocks and were resuspended at 5 x 10^6 cells/ml in 8% DMSO (Dimethyl Sulfoxide) / FCS (v/v) and 1ml was added per cryovial (Nalgene, USA). Vials were frozen by reducing the temperature by 1°C per minute to a temperature of -80°C by placing the vials in isopropanol containers, at which point cells were transferred to liquid nitrogen storage (-270°C).

2.2.2 Puromycin antibiotic selection kill curve

An antibiotic resistance kill curve was set up determine the minimal concentration of puromycin required to kill un-transfected CHO (Chinese Hamster Ovary) cells. 2 x 10^5 cells were seeded into six 100mm x 20mm cell culture dishes (Corning, NY, USA) and 10ml DMEM, 10% FCS was added per plate. Puromycin (Sigma) was added at varying concentrations: 0, 2.5, 5, 7.5, 10 and 12.5μg/ml. The cells were incubated at 37°C for 10 days and the selective media was replaced every three days.

Cell viability was observed in order to assess the minimal concentration required to induce massive cell death at 5 days leading to complete cell death within two weeks. 10μg/ml was determined to be the optimal selection concentration for this cell line and was then used during antibiotic selection of pApex DNA transfected cells.

2.2.3 G418 Sulphate antibiotic selection kill curve

2 x 10^5 CHO-SF cells were cultured in 10cm cell culture dishes in 10ml of SF media containing titrated concentrations of G418, 0, 50, 100, 200, 300, 400 500, 600, 700, 800, 900, and 1000 μg/ml. Cells were cultured for two weeks replacing medium every three days to remove non-viable cells. 500μg/ml antibiotic selection produced 100% killing of cultured cells post two week culture while 600μg/ml concentration prevented normal cell growth. 500μg/ml was chosen as optimal antibiotic concentration to maintain selective pressure on G418 resistant Signal pIgplus transfected cell lines.

2.2.4 Lipofectamine plus transfection of cells with plasmid DNA

Target cells were harvested and 1 x 10^5 cells were re-seeded per well in a 6 well tissue culture plate (Corning). 5 ml of cell specific media was added per well and the cells were incubated overnight at 37°C in order to obtain approximately 60-80% confluency at the time of transfection. The DNA was pre-complexed by mixing 1.5μg DNA [pApex-3P vector containing wild-type DAF (CD55) sequence donated from Bruce Loveland (Austin Research Institute, Heidelberg, Australia)] or respective plasmid, in 100μl of serum free media, with lipofectamine, (Invitrogen, Paisley, UK), 8μl diluted in 100μl serum free media, and incubated for 15 minutes at room temperature. Four separate DNA concentrations are used to determine optimal concentration for high expression levels (0.5-2.0μg DNA). The transfection liposomal/DNA complex was mixed with 800μl of serum free media. Cells were washed with serum free media and incubated with the lipofectamine complex for 16 hours at 37°C with 5% CO_2. 5ml cell specific media containing 10%FCS was added to the cells and incubated overnight at 37°C. Fresh media was applied to the cells which were continually cultured for a further 48hours. The media was again changed with the addition of plasmid specific antibiotic (10μg/ml puromycin for Apex plasmid). Antibiotic selection maintains growth of successfully transfected cells

expressing the relative antibiotic resistance gene. The cells were grown for several days under continuous selective pressure (as assessed by antibiotic kill curve analysis for each cell line) and assessed by flow cytometry using BRIC 216 [Anti-CD55 (SCR3 specific)], for expression of CD55 or via ELISA screening of cell supernatant for the presence of expressed protein.

CD55 positive cell clones were expanded, under selective pressure, and sorted by FACS with the aid of Dr. Adrian Robbins, University of Nottingham. High expressing clones were then utilised for immunisation. **Figure 2.1** displays vectors with their respective antibiotics.

Figure 2.1: Summary of vectors and their appropriate antibiotic selection

Plasmid	**Antibiotic resistance**
pApex-3P	Puromycin
Signal pIgplus	G418
pLXSN retroviral vector	G418

2.2.5 Calcium phosphate transfection of cells with plasmid DNA

The ProFection mammalian transfection system (Promega) was used as per the manufacturer's guidelines as an alternative to liposome based transfection protocols. 1 x 10^5 cells were seeded with 4ml of culture media into each well of a six well tissue culture plate. Plates were then incubated at 37°C with 5% CO_2 for 16 hours until cells reached between 50 and 80% confluency.

2 hours prior to cell transfection culture media was aspirated and replaced with fresh. Kit components, 2M calcium chloride, endonuclease free water and 2 x hepes buffered saline (HBS) were all thawed at room temperature. 6µg of plasmid DNA was combined with 112µl of sterile deionised water, and 18µl of 2M calcium chloride was vortexed with 150µl of 2 x HBS in a 1.5ml eppendorf tube. Then the DNA and $CaCl_2$ solutions were mixed drop-wise before incubating the combined solution for 30 minutes at room temperature. The transfection mixture was again vortexed prior to addition to target cells, drop-wise, gently swirling the plates. The cells were then incubated for 3 hours at 37C with 5% CO_2. The cells were then washed in sterile PBS and again in complete cell culture media. The cells were then fed with 4ml complete media and incubated at 37°C with 5% CO_2 for 48 hours. Appropriate antibiotic was

then applied to the cells for selective expansion of successfully transfected cells. Selection was maintained, changing media every three days for a further 14 days to establish stable transfected lines.

Transfection efficiency can be increased by the addition of 25μM chloroquine to the DNA/$CaCl_2$/HBS transfection mixture. A glycerol shock procedure may also be incorporated into the protocol, although particularly sensitive cells may be critically damaged. Following the three hour incubation with the transfection reagents, cells can be washed in PBS and then 1ml of 15% glycerol (in HBS) added for 2 minutes. This solution is then aspirated and cells washed in HBS prior to addition of complete media and cells incubated as standard. As an alternative to glycerol, 10% DMSO diluted in PBS can be used for the same duration.

2.2.6 Transfection of eukaryotic cells with β-galactosidase

As a control experiment for the determination of optimal transfection conditions specific to cell types, a β-galactosidase expressing plasmid can be used for the quick determination of successful protocols. The standard transfection methods can be used varying incubation times and DNA concentration. 48 hours following the transfection protocol cells are fixed by incubating cells with 2ml of 4% paraformaldehyde (Sigma) diluted in PBS, at 4°C for 5 minutes. Cells are then washed twice in cold PBS prior to enzyme activity assessment by the addition of 2ml Xgal staining solution and incubating the cells at 37°C with 5%CO_2 for between 30 minutes and 34 hours. Cells successfully transfected with the β-galactosidase plasmid will stain blues and can be visualised by light microscopy. Transfection efficiency can be calculated by counting all the cells in a viewed area, noting the number of positive, blue, cells. Efficiency is calculated as a percentage of positive cells within the total cell population.

X-gal staining solution contains 1 volume of x-gal stain [20mg/ml X-gal in dimethylformamide (DMF)] and 19 volumes of X-gal buffer (80mM Na_2HPO_4, 20mM NaH_2PO_4, 1.3mM $MgCL_2$, 3mM K_3FeCN_6 and 3mM K_4FeCN_6). The staining buffer is then 0.2 micron filter sterilised prior to use. All components are obtained from Sigma.

2.2.7 Viral transduction of eukaryotic cells

The retro-X transduction system (Clontech, BD biosciences, Palo Alto, CA, USA) utilises the production viral particles expressing the desired DNA sequence for high efficiency transduction of target cell lines.

The PT67 packaging cell line, which is able to package retroviral RNA's into infectious particles without the concomitant production of replication-competent virus, was transfected with the pLXSN retroviral vector into which had been cloned the full length CD55 DNA sequence. Successful transfected cells were cultured under G418 antibiotic selective pressure. Transfected PT67 cells express infectious replication incompetent retroviral particles containing the CD55 sequence into the cell culture supernatant.

Retroviral procedures were carried out in a designated laboratory within a class II safety cabinet, adhering to bio-safety level 2 guidelines.

2 x 10^5 PT67 cells were seeded onto 100mm tissue culture plates with 10ml of 10% FCS, 1% penicillin and streptomycin in DMEM media the day prior to transfection. Medium was aspirated and replaced with 10ml of fresh two hours prior to the transfection procedure. All transfection reagents, 2M calcium chloride, endonuclease free water and 2 x HBS were all thawed at room temperature. 10µg pLXSN/CD55 DNA was combined with 418µl of sterile deionised water, 62µl of 2M $CaCl_2$ and 500µl of 2 x HBS were combined in an eppendorf and vortexed. The DNA and $CaCl_2$ solutions were combined (DNA drop-wise to salt solution) and the mixture was incubated at room temperature for 30 minutes. The transfection mixture was vortexed prior to the drop-wise addition to the target cells, which were then incubated at 37°C with 5%CO_2 for 16 hours. The cells were then washed twice in complete media, which was then replaced with 10ml of complete media and the cells were incubated for a further 48 hours at 37°C. 500µg/ml of G418 antibiotic was then added to the cells in fresh media and the culture was maintained for a further 14 days, regularly replacing the culture media with fresh. Selective pressure was maintained in order to produce a stable transfected cell line. Antibiotic selective pressure was removed following the 14 day culture and cells were re-seeded in T175 tissue culture flasks with 50ml of complete media. The cells were grown for a further 48 hours to enable collection of virus particles within the culture supernatant. The collected supernatant

was collected and passed through a 0.45µm polysulphonic filter and polybrene (Sigma) was added to the supernatant at a concentration of 4µg.ml.

The day before transduction, 7 x 10^5 target cells were seeded into 100mm tissue culture plates with 10ml of complete culture medium and incubated for 16 hours at 37°C with 5% CO_2. The target cells were overlaid with viral supernatant and incubated for 12 hours at 37°C with 5% CO_2. The supernatant was aspirated from the target cells and the media was replaced with viral supernatant and incubated for a further 12 hours. The supernatant was aspirated and replaced with cell specific complete culture media and incubated for 48 hours at 37°C with 5% CO_2. G418 antibiotic was then applied for selection of successfully transduced cells. 600µg/ml was determined as the optimal concentration for use with Colo 205 cells.

2.2.8 Analysis of CD55 expression

Expression of CD55 by transfected or transduced cell lines was assessed by indirect labelling of cells with CD55 specific antibodies and analysed via flow cytometry. 5 x 10^5 cells were labelled and analysed per sample and all experimental procedures were completed in triplicate. 791T, Colo205 and CHO DAF cells were labelled with 100µl of 5µg/ml primary antibodies or 100µl diluted serum (1/1000 and 1/10000) or 50µl hybridoma supernatant. Controls were set up as unlabelled cells and cell without primary antibody. All samples were incubated for 1 hour at 4°C (on ice), and then samples were spun at 13000 rpm for 20 seconds to form a cell pellet. Cells were resuspended in 300µl of PBS and this wash process was repeated 3 times. Cells were then resuspended in 400µl secondary antibody [1/400 FITC-conjugated rabbit anti-mouse antibody (DAKO, A/5 Denmark)] diluted in PBS. Cells were incubated for 1 hour at 4°C and cells were then washed 4 times in PBS. The labelled cells were then resuspended in 300µl of 1 x Cellfix (BD Biosciences, Sunnyvale Ca. USA), containing 1% paraformaldehyde. Cells were either read immediately on the FACScan (Becton Dickinson), or stored overnight at 4°C and read the following day. For flow cytometry analysis, cells were transferred to FACS tubes (BD).

2.2.9 Flow Cytometry

Fluorescein (FITC) is excited at a wavelength of 488nm and collected via a 10nm bandwidth pass filter FL-1 (detection maximum = 518nm). Before samples are read,

forward and side scatter are set at the correct voltages in order to view them in a scatter plot. Utilising unlabelled cells, a main population can be gated so that outlying, dying or uncharacteristic, cells will be excluded from sample data. The voltages are set so that unlabelled cells produce a mean linear fluorescence value of 10 on a log scale. This value of M.L.F is calculated by determining the average fluorescence emitted by each cell compared to a total count of 5000 cells. This value is then said to be the auto-fluorescence of the cell populations, allowing comparison with cells possessing bound, labelled antibody on their surface. The FACScan (Becton Dickinson) is then used to measure the M.L.F values of experimental Cell samples and the results are displayed and analysed on CellQuest software on an Apple Macintosh™ computer.

2.2.10 Standard binding analysis of cell surface markers

Standard indirect or direct labelling of cells was achieved by following the protocol as stated in **section 2.2.8**. Primary antibodies are coated onto target cells and FITC conjugated secondary are bound to the primary for analysis by flow cytometry.

2.2.11 Isolation of CD55 expressing cells

Cells were sorted into sub populations at the Department of Immunology, Queens Medical Centre, Nottingham by Dr Adrian Robins using the EPICS Altra flow cytometer (Coulter). 1 x 10^7 cells to be sorted were labelled as per **section 2.2.8** and were resuspended in 10ml of PBS. The flow cytometer was then used to analyse the cells and relative levels of expression were identified. With the cytometer, cell populations expressing the greatest levels of CD55 were gated and collected. These cells were then washed in media and re-seeded on tissue culture flasks.

2.2.12 Isolation and cloning of cells transfected for protein/antibody production

Transfected cells were occasionally difficult to maintain for stable production of recombinant proteins or confirmation of expression for clonal expansion was required, as in the case of hybridoma production. Cells were washed in complete media and seeded in 96 well plates at titrating concentrations to obtain single cells per well. These cells were then cultured at 37°C with 5% CO_2 for the successful expansion of monoclonal cell populations. Cells were cultured for approximately 5

days and were then directly harvested or supernatants collected for assessment via FACscan binding assay or by ELISA protein assessment. Positive cells were then transferred to 6 well tissue culture plates before re-assessment and transfer to flasks for large-scale production.

2.2.13 Production and purification of recombinant proteins and antibodies

Eukaryotic cells successfully transfected with DNA constructs (plasmids), were cultured in complete cell specific media containing antibiotics specific to the plasmid present, in order to maintain selective pressure until a stable line is produced. Optimal antibiotic concentration was previously determined through the use of relative antibiotic kill curve analysis. Successful transfectants and protein expression was determined via ELISA screening of cell supernatants (**section 2.3.4**), and positive cells were re-seeded in T75 culture flasks. Selective pressure was maintained and cultures expressing the greatest levels of protein were transferred to T175 flasks, for large scale protein production. CHO SF cells were cultured in 100ml of complete media (serum free) at 37°C with 5% CO_2, every four to five days cells were harvested, supernatant pooled and 0.2micron filtered (stored at 4°C) and cells re-seeded. Cell stocks were made (**Section 2.2.1**) and stored at -80°C.

Culture supernatant containing expressed protein was stored until sufficient quantities were obtained for purification (~1 litre). Protein A Sepharose columns (Invitrogen, ,UK) were used to bind the protein via the human IgG (Fc) tail and were also used to purify murine antibodies. A standard glycine release protocol was followed for antibody and protein purification. **Figure 2.2** displays standard protein purification reagents.

Figure 2.2: Reagent composition for protein purification

Reagent	Composition
100mM Glycine –HCL pH 2.7	0.75g of Glycine 100ml of distilled water Adjust pH to 2.7 with 5M HCL
1M Tris-HCL	0.303g of Tris base 25ml distilled water Adjust pH to 9 with 5M HCL
Na_2HPO_4 (Buffer A)	3.04g Na_2HPO_4 100ml of distilled water
NaH_2PO_4 (Buffer B)	2.76g of NaH_2PO_4

	100ml of distilled water
Buffer C (20mM)	Add buffer B to buffer A until pH = 7.4

All components obtained from Sigma, UK, and all reagents 0.2micron filter sterilised.

10ml of buffer C ran over protein A column at speed of 1ml per minute to equilibrate column at 4°C (flow mediated with the use of vacuum pump) . Supernatant containing either antibodies or recombinant protein was then ran over the column at 4°C at a speed of 1ml per minute for a duration long enough to allow complete cycling of media at least two times through the protein A sepharose. 50ml of buffer C was then passed over the column to remove residual supernatant and balance the pH. A 500µl sample of run off buffer C was kept to determine whether any protein was lost during the wash procedure. 20ml of glycine was slowly added, preventing disruption of the protein A, and allowed to run through the column at 1ml per minute. 1ml fractions of eluted protein and glycine were collected in 1.5ml eppendorf tubes containing 100µl of Tris-HCL. 20ml of buffer C was then ran over the column to equilibrate the column and if the column was to be stored PBS containing 0.05% sodium azide was applied to the protein A prior to storage at 4°C. The collected samples were analysed by spectrophotometry and protein concentrations were determined by reading optical density readings at 280nm, blanked against a sample containing Tris-HCL and glycine. The samples containing protein were pooled and injected into a Slide a Lyze cassette (Pierce), which was placed in 500ml of sterile PBS and samples were dialysed at 4°C for 16 hours. Purified protein was then quantified by spectrophotometry and BCA protein assay (**section 2.2.14**) and assessed for viability via ELISA screen (**section 2.3.4**).

2.2.14 BCA protein quantification assay

Protein concentration was determined via spectrophotometric analysis, reading absorbance values of diluted samples at a wavelength of 280mn. Concentrations were confirmed using the BCA protein assay reagent kit (Pierce, Rockford, Illinois, USA) following manufacturer's guidelines. A standard curve was set up using 10 serial dilutions of BSA (bovine serum albumin) diluted in PBS. 10µl of protein samples and BSA standards were transferred to a 96 well plate, in triplicate and working reagent was prepared, combining 400µl of 4% copper II sulphate with 9.6ml bicinchoninic acid. 200µl of the working reagent was added to each well and the

plate was incubated at 37°C for 30 minutes. The plate was agitated and absorbance of samples was measured at 550nm. A standard curve of BSA concentration versus O.D. measurements was plotted and values extrapolated for test protein samples.

2.3 Antibody, sera and protein assessment

2.3.1 Standard enzyme linked immunosorbent assay (ELISA)

Buffer	Composition
Blocking buffer	1% bovine serum albumin (BSA) diluted in sterile phosphate buffered saline PBS
Wash buffer	0.05% Tween 20 in PBS
Antibody buffer	0.1% BSA in wash buffer
Citrate phosphate buffer	4.53g citric acid 4.06g sodium hydrogen phosphate Make up to 500ml in distilled water and adjust to pH 4.0
ABTS developing solution (2, 2'-azino-bis (3-ethylbenthiazoline-6-sulfonic acid)	66µl ABTS 13µl hydrogen peroxide 5 ml citrate phosphate buffer

Reagents supplied by Sigma Aldrich

2.3.2 Screening of Sera from peptide and purified CD55 immunisations via ELISA

Peptides and carriers (Ovalbumin and BSA) were diluted to 1mg/ml concentration in PBS and the two solutions were mixed together. Glutaraldehyde (Sigma) was added to a total volume concentration of 0.2%. The solution was mixed at room temperature for one hour and then dialysed twice against PBS in a 'slide-a-lyzer' cassette (Pierce). Conjugated peptides and purified CD55 were used to coat 96 well plates at a concentration of 5µg/ml (diluted in PBS), 100µl per well, all wells were performed in triplicate. Plates were incubated at 4°C overnight and peptide was flicked from plates, which were washed two times with PBS 0.1% Tween-20 [(Polyoxyethylenesorbitan monolaurate) (Sigma)]. Plates were blocked with 5% (w/v) Bovine Serum Albumin (Sigma; Basingstoke, UK) in PBS tween, 100µl per well and incubated for 1 hour at room temperature. Blocking solution was flicked from plates, which were then washed 3 times in PBS tween. 100µl of primary antibody were added per well (diluted serum 1/100, 1/1000, 1/5000, 1/10000, 1/100000 or 5µg/ml commercial antibody (Table B). For positive control of peptide

ELISA, 5µg/ml 105/AD7 antibody used to coat plate and 791T/36 antibody used as primary. Plates were incubated for 1 hour at room temperature and plates were flicked and wells washed 3 times with PBS tween. 100µl per well of secondary horseradish peroxidase conjugated anti mouse antibodies were added (1:400 PBS) and incubated for 1 hour at room temperature. The plates were washed 5 times in PBS tween and 100µl per well of ABTS (2, 2'-azino-bis (3-ethylbenthiazoline-6-sulfonic acid) (Sigma) substrate was added, which emits green light at a wavelength of 405nm. This substrate system requires 15ml of buffer containing 40mM Citric acid and 30mMNa_2HPO_4 at pH 4.0, with 0.08% H_2O_2 and 200µl ABTS. Plates were incubated for 15 minutes at room temperature prior to optical density analysis on a plate reader (Life Sciences, Basingstoke, UK).

2.3.3 Whole Cell Elisa

791T and Colo 205 cells were removed from culture flasks by incubation with 10ml Trypsin/EDTA (Sigma) for 10 minutes at 37°C. Suspended cells were then washed with complete media, centrifuged 1200 rpm for five minutes and supernatant was aspirated away and cells resuspended in appropriate volume of media for counting. 5 x 10^4 cells were added per well on a 96 flat bottom plate in a total volume of 100µl complete media. Plates were incubated at 37°C overnight. Media was aspirated off and cells were fixed with 100µl/well 0.5% gluteraldehyde (Sigma) in PBS for 15 minutes at 37°C. Cells were washed 2 times in PBS (100µl per well) and blocked with 100µl per well 5% BSA. Protocol continued as for peptide screen.

2.3.4 ELISA screen of SCR 1-3 Fc recombinant protein

Screening for the presence of recombinant protein within the supernatant of transfected cells was used to determine the success of transfection protocols and continually, for assessment of continued protein production in stable lines. 96 flat well plates were coated with 100µl of 5µg/ml CD55 specific antibodies BRIC 216 and 791T/36. Plates were incubated for 16 hours at 4°C to enable optimum binding. Plates were then washed three times in wash buffer and 100µl of cell supernatant was added in triplicate samples. Control wells were set up omitting primary antibodies. Plates were incubated for 1 hour at room temperature, supernatant discarded and plates washed three times with buffer. 100µl of 1/1000 dilution of goat anti human

(Fc specific) antibodies conjugated to horse radish peroxidase were added to all wells and plates incubated for 1 hour at room temperature. Antibody was again discarded and wells were washed five times with appropriate buffer. Plates were blotted dry and 100µl ABTS developing solution was added per well. Plates were developed in the dark for 15 minutes at room temperature and then analysed by reading the optical density at 405nm on an Anthos htII plate reader (Anthos Labtec Instruments, Salzburg, Austria).

2.3.5 Analysis of antibody responses generated in response to DNA immunisation

Assessments of murine antibody responses to DNA immunisation were required to obtain the titre of responses generated and the relative specificity. Early analysis of ELISA experiments following standard protocols indicated that direct coating of plates with recombinant protein significantly reduced binding of CD55 specific antibodies. Therefore, a capture assay protocol was developed utilising a primary well coat of goat anti human Fc antibody in order to orientate the recombinant protein. 100µl of 5µg/ml anti-human IgG Fc specific antibody was added per well on a 96 flat bottom plate and incubated for 16 hours at 4°C. Plates were washed in appropriate buffer three times and 100µl of blocking buffer was added per well and incubated for a further 16 hours at 4°C. Blocking solution was discarded and plates were washed in wash buffer three times and flicked dry. CD55 Fc fusion protein was then bound to the plates by the addition of 100µl per well of 5µg/ml of target protein. For specificity assessment, 100µl of 5µg/ml of human IgG antibodies (Sigma) were also added per well as an assessment of anti-human Fc responses (control wells) with the total anti sera. Plates were then incubated for 1 hour at room temperature and excess protein removed. Plates were again washed in buffer and flicked dry. Serial dilutions of anti-sera were prepared at concentrations of 1/100, 1/1000, 1/5000 and 1/10,000, dependent upon the total volume of sera available, diluted in sterile PBS. 100µl samples were added to wells, in triplicate, and incubated at room temperature for 2 hours. CD55 specific antibodies (791T/36 and BRIC 216) were used as positive controls and background recognition was assessed with the use of sera from syngeneic un-immunised mice. Sera were discarded and plates washed in appropriate buffer five times and plates were flicked dry. Rabbit anti mouse HRP conjugated antibody (DAKO, Glostrup, Denmark) was used at a dilution of 1/1000 (PBS) and 100µl were

added per well. Plates were then incubated for 1 hour at room temperature before washing all wells with buffer five times to successfully remove non specific peroxidase activity. The ABTS substrate system was used for detection of activity and plates were analysed at 405 nm as per standard ELISA protocol.

2.3.6 Assessment of antibody isotype ratios developed in response to DNA immunisation

The principle for this assessment was based on the antibody screen ELISA protocol (**section 2.3.4**). To incorporate the difference in relative affinities of antibody isotypes, a standard curve was developed incorporating CD55 specific IgG_1 and IgG_{2a} isotype antibodies. The standard Fc capture ELISA (**section 2.3.5**) was utilised and BRIC 216 and IA10 clone antibodies were serially diluted at concentrations of 10, 1, 0.03, 0.01, 0.003, 0.001, 0.0003 and 0.0001µg/ml and 100µl per well of these were applied to the orientated SCR 1-3Fc fusion protein. Plates were then incubated for 1 hour at room temperature prior to three washes in appropriate buffer. Plates were flicked dry and goat anti- mouse HRP conjugated antibodies (Serotec), specific to mouse IgG_1 and IgG_{2a}, were added at 1/1000 dilution, 100µl per well and plates were incubated for 1 hour at room temperature. Results for the assay were carried out following standard procedures using the ABTS substrate system. Absorbance readings at 405nm were then plotted against antibody concentrations for the production of a CD55 specific, isotyped antibody standard curve. The standard curve was then utilised to extrapolate values for assessed serum obtained from immunised mice. Mouse sera were screened as per **section 2.3.5**, using diluted sera at concentrations of 1/100, 1/1000 and 1/5000 (all in triplicate). The sera screen was changed by the addition of goat anti mouse isotype specific HRP conjugates, as for the standard curve. Plates were developed and analysed as per standard ELISA development.

2.4 Peptide Immunisations: Development of monoclonal antibodies

2.4.1 Isolation of splenocytes

Mice are sacrificed as per schedule 1 protocol (cervical dislocation) and sprayed with 70% ethanol to sterilise working area. Using several sets of sterile dissecting instruments, the spleen is removed from the mouse, excess fat and connective tissue is

removed and the spleen is transferred to a sterile 100 mm Petri dish. 5ml of serum free RPMI 1640 is added to the dish and the spleen is washed with the media using a syringe and 25-guage needle. The media is passed through the spleen several times and finally the spleen is homogenised with sterile forceps. The homogenate is passed through sterile gauze into a universal 25ml tube, total volume is increased to 25 ml with serum free RPMI, and the tube is centrifuged at 1000rpm for 10 minutes. The media is aspirated off, leaving approximately 1ml remaining in the cone of the tube, and cells are resuspended in 5 ml serum free media and counted using trypan blue (Sigma, UK).

2.4.2 Collection of large quantities of sera from immunised and naïve mice

Cardiac blood was collected using insulin syringes [microfine 11-100 insulin 12.7mm syringe, 1ml capacity (Becton Dickinson, France)] at the time of splenocyte collection.

2.4.3 Collection of Rat Peritoneal Exudate Cells (PECs)

Rat peritoneal exudate cells are used as growth support for hybridoma development, supplying growth factors and allowing presence of macrophages to remove any cell debris. A rat is sacrificed as per schedule 1 protocol and using a 20ml syringe and a 27 gauge needle, 20ml of RPMI 1640 media (Sigma, UK) is injected into the peritoneal cavity. The media is aspirated to collect PE cells from within the cavity, the media is withdrawn and cells are collected by centrifugation for 5 minutes at 1200rpm. The cells are resuspended and seeded over a 96 well, flat bottom plate at 2 x 10^5 cells per well. The rat PECs are placed in 200µl total volume RPMI 1640 containing 10% foetal calf serum (Sigma, UK), and grown for approximately 5 days at 37°C, 5% CO_2.

2.4.4 Fusion of Mice splenocytes with NSO myeloma cells

Mice producing the highest sera titres are chosen to generate antibody-producing hybridomas.

NSO cells are harvested, resuspended in serum free RPMI and counted for fusion with splenocytes. Cells are combined in a ratio of 1: 5 (NSO: Splenocytes) eg. (2 x 10^7 : 1 x 10^8) in a universal tube and centrifuged for 5 minutes at 1200rpm. The supernatant is aspirated off and the combined cell pellet is resuspended in 800µl PEG

[Polyethylene Glycol (Sigma, UK)], gradually over 1 minute. The PEG breaks the lipid membranes and allows fusion of the cell populations. The cells are agitated for 1 minute and then 1 ml of serum free RPMI 1640 is added over 1 minute while continuing to agitate. A further 5ml of S/F media is added to the cell suspension over a minute while continuing to agitate and finally the volume is slowly increased to 25ml with S/F media. The suspension is centrifuged for 5 minutes at 1200rpm and the supernatant is removed. The cells are resuspended in15ml hybridoma media [RPMI 1640, 10%FCS plus HAT supplement) Hypoxanthine Aminopterine Thymidine)], Sigma, UK. The cell suspension is spread evenly across a 96 well plate, containing rat PECs, and the cells are incubated at 37°C for approximately 8 days, by which time successful hybridomas should begin to have grown through. Cells are re-fed approximately every two days with fresh hybridoma media. Following 8 days supernatants can be collected and analysed for the presence of antibodies. Positive wells are harvested, washed in complete media and spread across 96 well plates in order to generate a monoclonal population of antibody positive clones.

2.5 General experimental procedures

2.5.1 Obtaining Normal Human Serum (NHS)

10ml of blood were collected from a minimum of three healthy donors in non-heparinised vacutainers (BD Biosciences, UK). Whole blood was stored at 4°C overnight to allow clotting of erythrocytes. Serum was withdrawn and pooled in a universal tube and centrifuged at 1500 rpm for 10 minutes in order to remove any remaining cells. Serum was again pooled and stored in 500µl fractions at 4°C for no longer than 2 weeks prior to use in complement deposition assays.

2.5.2 Complement Deposition Assay

Target cells (791T), were harvested and washed in complete media and PBS twice. Cells were resuspended in 1 ml of PBS and counted using a haemocytometer, and resuspended at a concentration of 1 x 10^6/ml. 300µl were then used per test sample (later to be divided into triplicate) and transferred to eppendorf tubes and primary antibodies were added at a concentration of 50µg/ml diluted n PBS. BRIC 216 and BRIC 220 (IBGRL) and 791T/36 were used as control antibodies to specific domains

of CD55. 100µl of hybridoma supernatant also added as test antibodies per sample (300µl added as to be split into triplicate). All tubes were agitated and incubated for 1 hour at 4°C. Control tubes also set up including completely unlabelled cells and complete range of samples with primary antibodies, which will not be exposed to complement. All tubes were centrifuged at 13000rpm for 20 seconds and supernatant aspirated from the cell pellets. Pellets were resuspended in 300µl of PBS and the centrifugation step was repeated. Cell pellets were then resuspended in 300µl of either 5% NHS (v/v in PBS) or PBS alone. Cells were then split into triplicate with 100µl total volume per sample. Cells were agitated and then incubated at 37°C for 2 hours, agitating briefly every 30 minutes. All tubes are centrifuged at 13000rpm for 20 seconds and then supernatants are aspirated from the cell pellets which are again resuspended in 200µl of PBS. This washing step is repeated a further two times with cold PBS. The cell pellets are then resuspended in 100µl 1/100 (v/v in PBS) polyclonal rabbit anti- human FITC conjugate (Dako, Denmark). The tubes are agitated and incubated at 4°C for 1 hour. Cells are washed a further three times with PBS before being resuspended in 200µl 1% cellfix (BD Biosciences, UK). Cells are stored at 4°C until they are to be assessed by FACScan.

2.5.3 SDS PAGE (Polyacrylamide gel electrophoresis)

The Miniprotean 3 gel kit (BioRad) was utilised for all SDS-PAGE following the manufacturer's guidelines. Glass back plates and cover plates were cleaned with 70% ethanol and once in acetone (Fisher Scientific) prior to being clamped into the gel-casting frame. 10ml of resolving gel was prepared and Acrylamide percentage was determined relative to the size of proteins to be electrophoresed. Ammonium persulphate and TEMED were applied to the solution immediately prior to application to the glass plates as it is this combination which catalyses the production of ions that cause the gel to polymerise. The solution was then applied between the plates, leaving approximately 2cm at the top of the plates for the stacking gel. 100µl of butanol was added to the surface of the gel, which was then left for 20 minutes to set. 2 ml of stacking gel was prepared, again leaving the addition of TEMED and APS until ready for application to the plates. The butanol was poured from the resolving gel and the surface was washed with distilled water, blotting residual water from the surface with blotting paper. The resolving gel was completed and applied to the

plates, inserting a clean well comb (containing the appropriate size and number of wells). Again the gel was allowed to solidify. The comb was removed and the wells washed with distilled water prior to transfer of the plates to the electrophoresis chamber. The central stage of the chamber was filled with running buffer and the external section was filled with enough buffer to cover a quarter of the plates. Samples to be electrophoresed were added to loading buffer in a 1:1 ratio, either reducing or non-reducing dependent upon protein analysis, and samples were denatured by a boiling step, heating to 95°C for 5 minutes. When a standard 10 well comb is utilised, 15µl of sample is loaded into the wells, along with 10µl of kaleidoscope pre-stained standards (BioRad) and if western blot analysis of proteins was to be carried out, 2µl of broad range biotinylated markers (BioRad), mixed with 2µl of reducing sample loading buffer, was also loaded into one well. All samples undergo the boiling procedure. A current of 40mA (powerpac 300, BioRad) was then applied to the electrophoresis chamber and allowed to run for approximately 1 hour or until the protein bands had sufficiently separated.

When proteins were to be assessed directly post electrophoresis, the gel was removed from the glass plates and the stacking gel was cut from the resolving gel section. The proteins were then fixed within the gel, to prevent diffusion, by coating the gel with fixative solution for a minimum of 30 minutes on a shaking table. Proteins were then visualised by the addition of Coomassie stain for a minimum of 2 hours, placed on a shaking incubator. Background stain was removed from the gel by treatment with de-stain solution for a minimum of 2 hours, placed on a shaking table. The protein gel was then washed in distilled water and 1% glycerol for 10 minutes on a shaking table. The gel was then dried in film. **Figure 2.3** displays standards SDS PAGE reagents.

Figure 2.3: Standard reagent composition for SDS PAGE

Solution	**Components**
1.5M Tris-HCL pH 8.8	54.4g Tris base in 200ml of distilled water. Adjust pH to 8.8 with concentrated HCL. Make total volume up to 300ml with distilled water
0.5M Tris-HCL pH 6.8	12.1g Tris base in 150ml of distilled water. Adjust pH to 6.8 with concentrated HCL. Make total volume up to 200ml with distilled water
10% (w/v) Sodium dodecyl sulphate (SDS)	5g of SDS in 50 ml of distilled water

10% (w/v) Bromphenol blue	1g of bromophenol blue in 1ml of distilled water
SDS-PAGE loading buffer	4.2ml distilled water 1ml 0.5M Tris-HCL pH6.8 0.8ml glycerol 1.6ml 10% (w/v) SDS +/- 0.4ml β2-mercaptoethanol (Reducing/Non reducing) 0.02ml 10% (w/v) bromophenol blue
10% (w/v) Ammonium persulphate (AMPS)	0.1g of AMPS in 1ml of distilled water Use Immediately
SDS-PAGE running buffer (1x)	15g of Tris base (25mM) 72g glycine (192mM) 5g SDS (0.1% w/v) Make up to 5 litres with distilled water
Transfer buffer	15g Tris base (25mM) 72g Glycine (192mM) Make up to 4 litres with distilled water Add 1000ml methanol (20% v/v)
Tris buffered saline (TBS) pH 7.6	20ml 1M Tris HCL pH 7.6 (20mM) 8g Sodium chloride (137mM) Make up to 1000lml with distilled water
TBS-Tween (TBS-T) (wash buffer)	0.05% (v/v) Tween 20 in TBS
30% acrylamide solution	Obtained from Severn Biotech Ltd, Kidderminster, UK
TEMED	Neat solution

All chemical components obtained from Sigma Aldrich.

2.5.4 Western Blotting

The anode (base plate) of the western blotter (Transblot SD, BioRad) was soaked with western transfer buffer). For each gel to be transferred to nitrocellulose membrane (Hybond C, Amersham Life Science), six pieces of filter paper and one piece of membrane matching the dimensions of the resolving gel were cut and also soaked in transfer buffer. Three layers of filter paper were placed on the anode with one piece of membrane placed on top. A resolving gel containing unstained electrophoresed protein samples was washed in western transfer buffer and then placed on top of the nitrocellulose membrane noting the correct orientation. All proteins run are of net negative charge and will move towards the anode. A further three pieces of soaked filter paper were placed on top of the gel and a glass rod was lightly rolled over the layers to ensure removal of any air bubbles. The top plate (cathode) of the western blotter was then washed in transfer buffer and fixed in place. A current of 150mA was passed through the plates for 1 ½ to 2 hours. The nitrocellulose membrane was

then washed in PBS and used for immuno-blotting or allowed to air dry and stored at -20°C. **Figure 2.4** displays standard western blotting reagent composition.

Figure 2.4: Standard Western Blotting reagents

Solution	Components
Towbin Transfer buffer	25mM Tris base (7.6g) 192mM Glycine (36.0g) 20% Ethanol (500ml) Make up to 2500ml with distilled water and adjust to pH 8.8
Fixative solution	25% Isopropanol (75ml) 10% Glacial acetic acid (30ml) 65% deionised water (195ml)
Coomassie staining solution	50% (v/v) Methanol 20% (v/v) Glacial acetic acid 0.0012% (w/v) Coomassie blue R-250 30% (v/v) deionised water
De-staining solution	5% Methanol 7% Glacial acetic acid 88% deionised water
Blocking solution (Dependent on potential cross reactivity of antibodies)	1% BSA (bovine serum albumin) in PBS 1% BSA plus 5% Marvel™ diluted in PBS

2.5.5 Standard Immuno-blotting protocol

Following transfer of electrophoresed proteins to nitrocellulose membrane blots were placed in either 1% bovine serum albumin (BSA, Sigma) or 5% Marvel™ milk powder, dependent upon cross reactivity of antibodies to be used in the detection step. Non specific binding sites were blocked for 16 hours at 4°C placed on a shaking table. Membranes were then washed in PBS 0.05% Tween 20, three times for 10 minutes at room temperature. Membranes were then incubated for 2 hours at room temperature with specific primary antibodies diluted in blocking buffer (dilution for optimum activity dependent on specific antibody, common dilution 1/1000). The membrane was then washed for 30 minutes in PBS Tween, changing the wash buffer every 5 minutes and rinsed a further three times (quick washes). Membranes were then placed in primary specific, horse radish peroxidase conjugated secondary antibody diluted as per data sheet for optimal activity in blocking solution for 2 hours at room temperature (1/2000 for rabbit anti mouse HRP), and placed on a shaking incubator. Biotinlylated standard lanes were treated with 1/1000 streptavidin HRP conjugate

(BioRad) diluted in 1% BSA, and incubated for2 hours at room temperature (under agitation). Membranes were then washed four times for 10 minutes in fresh PBS Tween. The chemiluminescence ECL detection system (Amersham Life Sciences) was used to detect antibody recognition of protein bands in a dark room. 2ml of ECL reagent (1.95ml solution A plus 50µl of solution B) was applied to each membrane and incubated at room temperature for 5 minutes. Excess reagent was then removed from the membrane, which was then wrapped in saran film, preventing incorporation of air bubbles. The nitrocellulose membrane was then placed in a developing cassette and covered with a piece of blue sensitive X ray film (GRI, Rayne, UK). Film was initially exposed for 30 seconds before development and fixation in photographic development agents. To obtain the clearest image, several pieces of photographic film were exposed to the membrane for varying times prior to development. The film was then washed in distilled water and allowed to air dry.

2.5.6 Assessment of RM1 antibody specificity to electrophoresed protein

The RM1 antibody was tested by western blot analysis against recombinant CD55 (SCR1-4-Fc. Harris CL 2000). Protein was electrophoresed on 8% SDS-polyacrylamide gel under non-reducing conditions and transferred to nitrocellulose (Amersham Life Sciences) for 1 ½ hours at 150mA in transblot apparatus (Bio Rad). The membrane was blocked for 24 hours at 4°C in 2% Marvel^{TM}, 3%BSA (Sigma) and probed with 2µg/ml anti CD55 antibodies, RM1, BRIC 216 (IBGRL) and 791T/36). Primary antibody was added at 1/1000 (v/v) and incubated at room temperature for 1 hour in 5% BSA, washed in PBS-Tween (0.1%) and then incubated with peroxidase conjugated rabbit anti-mouse secondary antibody for 1 hour at RT. The blot was washed three times prior to developing with ECL reagent (Amersham Life Sciences,).

2.5.7 Analysis of cytokine production within mouse sera and culture supernatant (Luminex assay)

Bioplex kits were obtained from Bio Rad for use with their standard assay procedure. A mouse cytokine/chemokine 10plex panel (#60-00006GK) was obtained for assessment of IL-2, IL-3, IL-4, IL-5 IL-6 IL-10, IL-12p40, IL-12p70, IFNγ and TNFα. The Luminex machine was calibrated daily following manufacturer's

guidelines prior to any subsequent use. 1 vial of mouse cytokine standards was reconstituted with 500µl of assay media (matching growth media of cultures cells) equating to 50,000pg/ml and incubated on ice for 30 minutes. The standards were then diluted to give the following concentrations; 32,000pg/ml, 8000pg/ml, 2000pg/ml, 500pg/ml, 125pg/ml, 31.3pg/ml, 7.8pg/ml and 1.95pg/ml, all standards were kept on ice. Beads were reconstituted by combining 1.25µl of beads per well (sample) added to assay buffer (30µl per sample/well) and the solution was stored on ice. Detection antibody was reconstituted by combining 0.625µl per sample with 15µl per sample of Diluent A (stored on ice). Streptavidin-PE was reconstituted by combining 0.313µl Strep-PE/sample with 31µl per sample of Assay buffer, the solution was stored in the dark on ice. The following staining procedure was carried out for the complete assay. A 96 well non-protein binding plate (Millipore, UK) was coated with 150µl per well of Assay buffer and then aspirated using a vacuum pump (as per manufacturer's guidelines), the plate being blotted dry to remove residual buffer. 25µl per sample of beads were added to the plate per sample and the supernatant was aspirated using the vacuum pump and the base of the plate was blotted dry. All wells were then washed twice (25µl/well) with wash buffer, aspirating all liquid with the vacuum pump. 25µl per well of both samples and standards were added to separate wells. Cell culture supernatant was used undiluted while serum samples were diluted (3:1) in mouse serum diluent as per manufacturer's guidelines. The plate was wrapped with foil and incubated at room temperature under agitation for 1 hour. Supernatant was aspirated and wells were washed three times in appropriate buffer as previously described. 12µl per well of detection antibody was added and the plate was again covered and incubated for a further hour at room temperature. All liquid was again aspirated and the plate was washed a further three times with wash buffer and blotted dry. 25µl of Strep-PE was added per well and the plate was covered and agitated for 10 minutes at room temperature for 10 minutes. The plate was aspirated and washed three times in buffer prior to the addition of 125µl/well assay buffer. The plate was agitated for 30 seconds prior to assessment of results on the Luminex machine. The standards were used to generate a standard curve enabling calculation of sample concentrations.

2.6 Development and analysis of DNA constructs

2.6.1 Standard Agarose gel electrophoresis

Standard 'Minigel' apparatus (Horizon 58' Gibco BRL, Invitrogen Ltd, Paisley, UK) was assembled following manufacturer's guidelines. 100ml of a 1% agarose gel was prepared in 1% Tris borate EDTA (TBE) buffer and heated in a microwave for ~60 seconds, until all the agarose had fully dissolved, with intermittent agitation to ensure complete mixing. The mixture was then cooled to ~50°C and Ethidium bromide was added at a concentration of 0.5µg/ml and gently swirled to ensure complete mixing. This was performed in a fume cupboard as per safety regulations. The gel was then poured into the minigel apparatus and an appropriate well spacer (comb) was inserted, dependent upon sample size to be used. The gel was then left at room temperature to allow the agarose to set. 1 x TBE solution was used as running buffer and sufficient quantity was applied to cover the complete gel. The gel was then allowed to equilibrate with the buffer for 10 minutes prior to removal of the comb.

DNA samples were combined with loading dye, 8µl sample plus 2µl dye, [15%FICOLL (Pharmacia Biotech Ltd, St Albans, Hertfordshire, UK), 0.25% xylene cyanole (Biorad, Hercules, California, USA), 0.25% bromophenol blue (Biorad)] all dissolved in water. Samples were mixed via pipette action and loaded into the gel wells. The gel apparatus was connected to an electric power supply (Pharmacia LKB-GPS200, Pharmacia, Peapack, NJ, USA) and a voltage of ~90 Volts was applied for ~1 hour to ensure sufficient movement and separation of desired DNA fragments. Analysis of electrophoresed samples was assessed by viewing on an ultraviolet trans-illuminator (dual density transilluminator, UVP, San Gabriel, CA, USA), If further band separation was required, the gel was placed in the electrophoresis equipment and electrophoresed for the desired time. Pictures of assessed DNA were obtained using a DS34 direct screen instant camera (Polaroid, UK)

2.6.2 High purity agarose electrophoresis

When high quality DNA was to be used post electrophoresis, for excision and restriction digest analysis and/or incorporation into DNA constructs, 1.2% NuSieve

agarose (Flowgen, Lichfield, UK) was used instead of the standard agarose. All other procedures were followed as per the standard protocol.

2.6.3 Single enzyme restriction digestion of DNA

The reaction mixture was set up in sterile micro centrifuge tubes with all components being stored and maintained on ice. Reaction components: 5µl DNA, 2µl restriction enzyme, 3µl enzyme specific reaction buffer and 20µl of sterile water. The reaction mix was then incubated at room temperature overnight at room temperature and dependent upon the enzyme used a heat inactivation step was incorporated (65°C for 25 minutes). All restriction digests were then analysed via electrophoresis. In some cases, further digestion of the DNA was required with other enzymes. The DNA was excised from the gel and phenol chloroform extraction was then performed (**Section 2.6.6**) and ethanol precipitation (**Section 2.6.7**) was carried and the DNA pellet was resuspended in 5µl of sterile water prior to further enzyme restriction.

2.6.4 Multiple restriction enzyme digestion of DNA

In cases where dual enzyme restriction digestion was required and buffer conditions for optimal enzyme activity were compatible, the double digest was performed in a single step. The reaction mixture was modified: 5µl DNA sample, 2µl restriction enzyme A, 2µl restriction enzyme B, 3µl restriction enzyme reaction buffer and 18µl sterile water. The protocol for single digestion was then followed. **Figure 2.5** displays restriction enzyme conditions.

Figure 2.5: Summary of conditions used for restriction enzymes

Plasmid	**Restriction Enzyme**	**Buffer**	**Heat Inactivation**	**Supplier**
PCRII-TOPO	*Xba I* *Not I*	3	65°C	New England Biolabs (NEB), USA
pCR3.1	*Kpn I* *Not I*	1 3	N/A 65°C	NEB
Signal pIgplus	*Hind III* *Not I*	2	65°C	NEB
pApex	*Bgl I* *Pvu II*	2	N/A	NEB
	Eco RI	U	65°C	NEB
	Bam HI	U	N/A	NEB

U = Unique restriction enzyme reaction buffer

2.6.5 DNA extraction from agarose gel

Electrophoresed DNA was visualised under ultraviolet light and appropriate bands were excised from the gel with a scalpel. The DNA was then obtained utilising the Wizard DNA cleanup purification system (Promega, Southampton, UK), following the manufacturer's standard protocol. The excised gel fragments were dissolved in 100µl 1M mannitol (Sigma) and 1ml of wizard cleanup resin at 65°C for 30 minutes. The DNA was isolated by passing the dissolved mixture through a cleanup filter which was then washed with 2ml of 80% isopropyl alcohol (BDH Ltd, UK) and eluted with 30µl of 70°C sterile water.

2.6.6 Removal of impurities from DNA samples via solvent extraction

In order to remove contaminants from DNA samples such as enzymes from previous digestions, proteins were denaturised via solvent extraction. Sterile phosphate buffered saline (PBS) was added to the DNA sample to increase the volume to 100µl. 50µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the DNA, the sample was 'vortexed' for 60 seconds and centrifuged at 13,000 rpm for 10 minutes in a micro-centrifuge. The upper aqueous phase was then transferred to a sterile micro-centrifuge tube, discarding the lower phase. 50µl of chloroform: isoamyl alcohol (24:1) was then added, 'vortexed' and re-centrifuged. The upper layer, containing the extracted DNA, was again transferred to a clean tube to carry out the ethanol precipitation procedure.

2.6.7 Ethanol precipitation of DNA

Following solvent extraction 0.1 volumes of chilled 3M potassium acetate pH 5.2, and 2 volumes of chilled 100% ethanol (BDH Ltd, Poole, UK) were added to the eluted DNA solution. This reaction was placed at -20°C for 30 minutes and centrifuged for 10 minutes at 13,000rpm in a micro-centrifuge. The precipitated DNA appears as a 'glassy' pellet and the supernatant was discarded. 200µl of chilled 70% ethanol was added and the centrifugation step was repeated. The supernatant was discarded and the DNA pellet was air dried to remove residual ethanol and resuspended in a desired volume of sterile water.

2.6.8 DNA quantification by spectrophotometry

Utilising a Genequant pro spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden), a cleaned quartz cuvette was used to analyse the DNA for quantification. A 'blank' measurement was obtained reading sterile water at a wavelength of 260nm. Dilutions of the DNA sample were set up in sterile water and their optical density was analysed at 260nm compared to the 'blank' result. The sample concentration is calculated using the formula $OD_{260} = 1 = 50\mu g/ml$ correcting for the dilution factor. 260nm/280nm readings were also obtained for protein purity.

2.6.9 TA Cloning

The Topo TA cloning kit (Invitrogen) utilises the pCRII-TOPO plasmid which incorporates the topoisomerase I enzyme which enables one step cloning of Taq polymerase amplified products into a plasmid vector. Reactions were set up using 4µl of PCR product, 1µl MgCl solution and 1µl pCRII-TOPO in sterile micro-centrifuge tubes and incubated at room temperature. 2µl of this product could then be used to transform competent bacteria as per **section 2.6.11**.

2.6.10 Ligation of DNA products into plasmid vectors

Reactions were set up: 1µl of restricted vector, 3µl insert (product), 1µl T4 DNA ligase (New England Biolabs (UK) Ltd, Hitchin, Hertfordshire,UK), 1µl 10 x buffer and 4µl sterile water in a sterile micro-centrifuge tube. The reaction mixture was then placed at room temperature overnight. A sample of the ligation reaction can then be assessed via agarose electrophoresis in order to obtain whether the insert has been successfully incorporated. Further restriction analysis of ligation products may be carried out in order to assess alternate products with their relative sizes, in order to obtain whether ligation is completely successful.

2.6.11 Transformation of competent bacteria with plasmid DNA

Several strains of competent bacteria were used throughout this research including DH5α (Gibco Life Technology, Invitrogen BV, Leek, The Netherlands), TOP10F (Invitrogen BV) and XL1-Blue (Stratagene Europe, Amsterdam, The Netherlands). 50µl aliquots of competent cells were stored at -80°C and individual aliquots were used per transformation. Cells were thawed on ice and transferred to a chilled

eppendorf tube. 1µl of plasmid or ligation reaction were added to the cells and gently mixed with a pipette tip. The cells were then incubated on ice for 30 minutes. pUc18 (Invitrogen) plasmid was used as a control transformation reaction and 1µl was used with 1 vial of competent cells. Cells were then heat shocked by placing the tubes in a 42°C water bath for 30 seconds and directly transferred to ice for a further 2 minutes. SOC media was heated to 37°C and 500µl were added to the transformed cells which were then placed in a shaking incubator (G25, New Brunswick Scientific Co Inc, Edison, New Jersey, USA) at 37°C for 60 minutes. The transformed cells were then placed on ice and 75µl of the cells were seeded onto an LB agar plate containing 100µg/ml ampicillin, applying selective pressure to only enable growth of successfully transformed cells (plasmids used contain an ampicillin resistance gene). Seeded plates were then incubated at 37°C for at least 16 hours. Colonies were then assessed for incorporation of plasmid DNA. Colonies were spiked with a pipette tip and used in a PCR reaction with appropriate DNA primer to obtain a PCR product, with non-transformed cell not producing a band as visualised by agarose gel electrophoresis.

2.6.12 Small-scale production of plasmid DNA

The Qiaprep spin miniprep kit (Qiagen Ltd, Crawley, UK) was utilised following the standard manufacturer's protocol in order to generate a maximum of 20µg of plasmid DNA. Colonies containing the desired plasmid were spiked with a wire loop and used to inoculate 5ml of LB media, containing 100µg/ml ampicillin. The media was then incubated in the shaking incubator for 16 hours at 37°C to allow culture of the selected bacteria. The culture was then centrifuged for 20 seconds at 13,000rpm in a micro centrifuge to obtain a bacterial cell pellet; approximately 200µl of the media was stored at 4°C for large scale production. Alkaline lysis of the cell pellet was performed using the reagents provided and the supernatant was passed through a Qiaprep column and centrifuged at 13,000rpm for 30 seconds. The column was then washed and the DNA, which binds to the column, was eluted by the addition of 50µl of 42°C EB buffer. DNA mini preparations were then used for further analysis in PCR and sequence determination.

2.6.13 Large-scale production of plasmid DNA

Once mini preparations of plasmid DNA have been sequenced, large-scale production of the DNA constructs was desired to make stocks and for further experimentation. Utilising the Qiagen maxi plasmid purification system up to 500µg of plasmid DNA can be generated. 5ml of ampicillin selective LB was inoculated with 200µl of stored culture or was inoculated with a plasmid positive colony. This culture was then incubated for 8 hours at 37°C in a shaking incubator. 1ml of this culture was then used to inoculate 500ml of ampicillin selective media. This larger volume was then split into two 1 litre flasks and incubated at 37°C for 16 hours (shaking). 500µl aliquots were collected for glycerol stocks and the remaining culture was centrifuged at 13,000 rpm for 10 minutes in order to obtain a bacterial pellet. The pellet was then used in an alkaline lysis step in order to release the plasmid DNA. Bacterial components of the lysates were then precipitated and filtered from the supernatant containing the target DNA, which was bound to a Qiagen-tip 500 column. The column was then washed and the plasmid DNA eluted. The DNA was then precipitated with isopropyl alcohol and washed with 70% ethanol. The DNA was air dried and resuspended in 500µl of endotoxin free water. DNA yield and purity was then determined via spectrophotometry (**Section 2.6.8**).

2.6.14 Production of endotoxin free plasmid DNA (Immunisation quality)

DNA plasmid constructs were to be used as DNA immunisations and due to this, removal of all bacterial endotoxin was required for a pure immunisation. Endotoxins/Lipopolysaccharides are cell membrane components produced in gram-negative bacteria and within humans and animals can activate components of the innate immune system, such as complement, and also inhibit immune cell transfection. Qiagen Ltd produces an endofree plasmid mega purification system, which can be used to generate up to 2500µg of endotoxin free plasmid DNA.

The standard protocol was followed which copies the maxi plasmid purification system except the filtered cell lysates are passed through Qiafilter mega cartridges and filtered eluate is mixed with ER buffer, that removes endotoxins before being passed through a Qiagen-tip 2500 column. The column was then washed and the DNA eluted as before. Isopropyl alcohol precipitation and ethanol cleaning steps are used as per the standard protocol, maintaining use of endotoxin free components. The

DNA pellet obtained was resuspended in 500µl of endotoxin free water and analysed by spectrophotometry.

2.6.15 DNA Sequence analysis

Sequence analysis was performed in the Department of Biochemistry, University of Nottingham. Plasmids were sequenced using the appropriate primers with the BigDye terminator cycle sequencing ready reaction (PE Applied Biosystems) and sequences were electrophoresed using the ABI prism 373A DNA sequencer (Applied Biosystems). **Figure 2.6** displays the primer pairs used for each plasmid vector.

Figure 2.6: Vectors and their respective primer pairs

Vector	Primer Pair	
	5’	3’
pCR3.1	T7	pCR3.1
pCRII-TOPO	M13 Forward	M13 Reverse
Signal pIgplus	T7	SIGR

Primer pairs used for amplification of multiple cloning sites in specific plasmids

2.6.16 Polymerase Chain Reactions (PCR)

Primer	Sequence (5’ to 3’)
T7	TAATACGACTCACTATAGGG
M13 forward	GTAAAACGACGGCCAGT
M13 reverse	GGAAAACAGCTATGACCATG
pCR3.1	TAGAAGGCACAGTCGAGG
Xa 5’	GATCCCATCGAAGGTGGTGGTGGTGGTGG
Xa 3’	CCACCACCACCACCACCTTCGATGGGATC
SIGR	CATGTGTGAGGTTTGTCACAAG

Primer sequences used for all PCR reactions, primers synthesised by the Department of Biochemistry, University of Nottingham, UK.

2.6.17 Standard reaction protocol

The standard reaction mixture consisted of 100pmol 5’primer, 100pmol 3’primer, 200µM dNTPs, 10µl of 10 x reaction buffer and 84µl sterile water, all prepared in a sterile micro-centrifuge tube. Either 20ng of template DNA or bacterial cells were

spiked from a colony containing the target plasmid. Negative control reactions were set up containing irrelevant primers and reaction mixes without template DNA. Each reaction was covered with a layer of mineral oil (50µl) to prevent evaporation of the reaction mixture. Tubes were placed in a PHC-3 thermal cycler (Techne, Cambridge, UK), and a program was set up to include optimal temperatures for annealing and extension steps. A 'hot start' was set up for optimal polymerase activity and denaturation of the target DNA, heating samples to 95°C for 5 minutes before addition of 1µl of Super Taq DNA polymerase (HT Biotechnology Ltd, UK). The reaction samples then underwent thirty cycles of, 94°C for 30 seconds, 55°C for 55 seconds and 72°C for 55 seconds. A final extension step of 72°C for 10 minutes was performed before final cooling to 4°C indefinitely. Successful generation of PCR products was determined by agarose gel electrophoresis (**Section 2.6.1**).

2.6.18 Site directed mutagenesis protocol for removal of factor Xa cleavage site from signal pIgplus plasmid

As signal pIgplus constructs were to be used for both *In Vitro* and *In* Vivo expression of recombinant proteins linked to a human IgG Fc region, the factor Xa cleavage site was removed from the plasmid to prevent cleavage of the Fc domain. This was achieved utilising the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, California, USA) following all manufacturer's guidelines. Primers were designed to replace a Cysteine with a Glycine, thus preventing formation of the cleavage site. 50ng of signal pIgplus plasmid DNA was added to 0.4µl 5' Xa primer, 0.4µl 3' Xa primer, 1µl dNTPs, 5µl of 10 x reaction buffer, 42.2µl sterile water and 1µl of pfu turbo, all placed in a micro-centrifuge tube. As per standard PCR protocol the sample was overlaid with 50µl of mineral oil. The tube was placed in a thermal cycler and the following program set up: 95°C for 30 seconds, 12 cycles of, 96°C for 30 seconds, 56°C for 1 minute, 69°C for 13 minutes and the final step was 4°C indefinitely. Methylated bacterial DNA (non-mutated) was then digested by the addition of 1µl of dpn1 enzyme and incubating the reaction mixture for 1 hour at 37°C. Chemically competent bacterial TOP 10F cells were then transformed with the mutated DNA plasmid as per **section 2.6.11**. Mini preparation of plasmid DNA was then performed (**section 2.6.12**) and retrieved DNA was sequenced for confirmation of the successful point mutation.

2.6.19 Site directed mutagenesis for incorporation of heteroclitic epitopes into the SCR 1-3 Fc constructs

To generate a DNA vaccine incorporating heteroclitic epitopes within the SCR 1-3Fc construct, identified epitopes needed to be inserted into the existing vaccine plasmid. The standard Quickchange II site directed mutagenesis kit was used to insert point mutations within the CD55 sequence following the manufacturer's guidelines. Primers were designed with base substitutions to incorporate the desired sequences and were used in the standard reaction mix and thermal cycling protocol. Primers were assessed for possible self-hybridisation and production of non-desirable products, annealing temperatures and percentage of GC oligonucleotides. 5µl of 10 x reaction buffer was combined with 10ng of template DNA, 125ng 5' primer, 125ng 3'primer, 1µl dNTP mix, 3µl quick change buffer and the reaction volume was increased to 50µl with sterile distilled water. 1µl pfu turbo (2.5U/µl) was added prior to use with the thermal cycling procedure. The standard cycling protocol is displayed within the table indicating alterations in the extension step relative the size of DNA to be amplified (1 minute per kb of plasmid DNA). The standard procedure was then followed for digestion of Methylated bacterial DNA. **Figure 2.7** displays primers designed for mutagenesis of incorporated epitopes.

Figure 2.7: Heteroclitic epitope primers

Primer		Sequence 5' → 3'	Cycles
Z69 (CD8)	Forward	CGG CTG CTG CTG CTG TTG CTG TTG TGC CTG CC	1 x 95°C 16 x { 95°C for 30 seconds 55°C for 1 minute 68°C for 14 min. 17s
	Reverse	GG CAG GCA CAA CAG CAA CAG CAG CAG CAG CCG	
Z143 (CD8)	Forward	CA CCA AAA CTA ACT TGC GTT CAG AAT TTA AAA TGG TCC ACA GC	1 x 95°C 16 x { 95°C for 30 seconds 55°C for 1 minute 68°C for 14 min. 17s
	Reverse	GC TGT GGA CCA TTT TAA ATT CTG AAC GCA AGT TAG TTT TGG TG	
Z158m (CD8)	Forward	CA GAT GTA CCT AAT GTC CAG CCA GCT TTG GAA GGC	1 x 95°C 16 x { 95°C for 30 seconds 55°C for 1 minute 68°C for 14 min. 17s
	Reverse	GCC TTC CAA AGC TGG CTG GAC ATT AGG TAC ATC TG	

Red bases represent substitutions used to incorporate point mutations within DNA sequence. Primers synthesised by the Department of Biochemistry, University of Nottingham, UK.

2.6.20 Bacterial cell culture

All bacterial cell culture was carried out using aseptic technique and following all guidelines for genetic manipulation risk assessment. All cultures were grown under selective conditions with appropriate antibiotics. Single colonies were obtained by the streaking of cultures/glycerol stocks over LB agar plates and these were stored for a maximum of three weeks at 4°C prior to incineration. In order to maintain stable cultures containing desired plasmids, all stock and DNA preparations were made with fresh colonies and cultures. **Figure 2.8** displays components of culture media used.

Figure 2.8: Media components for bacterial cell culture

Medium	Components
Luria Bertani (LB)	10g bacto-tryptone (DIFCO, Becton Dickinson) 5g bacto-yeast extract (DIFCO) 10g sodium chloride (Fisher Scientific) Make up to 950ml deionised water pH 7.0
LB Agar	As per LB media plus 15g agar (DIFCO)
SOB	20g bacto-tryptone 5g bacto-yeast extract 0.5g sodium chloride Make up to 950ml deionised water Add 10ml of 250mM potassium chloride (BDH Ltd) Adjust pH to 7.0 Final volume made up to 1 litre. Immediately prior to use add 5ml of sterile 2M magnesium chloride
SOC	As per SOB medium plus 20mM glucose (20 ml of sterile 1M glucose solution added post sterilisation

All media autoclaved for 20 minutes at 15lb per in^2

2.6.21 Glycerol stock preparation of bacterial cultures

LB media cultures (containing 100µg/ml ampicillin), which had been used for 'miniprep' and 'maxiprep' generation of plasmid DNA were used to make cell stocks. 500µl aliquots of culture were mixed with 500µl sterile 30% (v/v) glycerol. Samples were placed in cryovials, labelled and stored at -80°C.

2.7 DNA immunisation: Analysis of cellular responses

2.7.1 Preparation of Lipopolysaccharide (LPS) blasts

Splenocytes were harvested from a syngeneic (per experiment) un-immunised mouse as per **section 2.4.1**. The cells were then washed in culture media and resuspended at a concentration of 1.5 x 10^6/ml. LPS was then added at a concentration of 25µg/ml with 0.05mM β-mercaptoethanol and 7µg/ml of dextran sulphate. Cells were then transferred to T175 culture flasks at a maximum volume of 50ml per flask. The cells were then incubated at 37°C in 5% CO_2 for 2 days.

2.7.2 Preparation of murine CTL effector cells

Splenocytes were harvested from immunised mice as per **section 2.4.1** were washed in culture media and resuspended at a concentration of 4 x 10^6/ml. 1ml aliquots were seeded per well in 24 well culture plates in complete media. Between 5 x 10^4 and 1 x 10^6 LPS blasts, which were irradiated with 20Gy from Gammacell Caesium^{157} source to prevent proliferation, were added per well of immunised effector cells. Total culture volume was 2ml of complete media. Prior to the addition of the irradiated blasts, they were incubated with 10µg/ml of target peptides, which mimic desired epitope targets (synthesised by Alta Biosciences, Birmingham, UK). The 24 well plates were then incubated at 37°C with 5% CO_2 for 6 days prior to assessment of peptide specific CTL activity as assessed by chromium release assay (**section 2.7.4**).

2.7.3 Preparation of target cells for CTL assay

T2 cells were passaged one day prior to the chromium release assay in order to maintain optimal cell viability. Cells were centrifuged at 12,000rpm for 5 minutes to produce a cell pellet. The cells were then washed in serum free RPMI media two times before re-suspension in complete media at a concentration of 1 x 10^6/ml. All counts were performed with trypan blue staining. 1ml aliquots were then pulsed with 100µg/ml of target peptides (immunising epitope) and 1.85MBq of sodium [^{51}Cr] chromate (Amersham Pharmacia biotech, Essex, UK) and incubated at 37°C in 5% CO_2 for 2 hours. The cells were then washed in complete culture media three times before counting with trypan blue and re-suspension at a concentration of 5 x 10^4 cells per ml.

2.7.4 Chromium release assay

The *In Vitro* stimulated murine CTL effector cells were pooled on day 6 of culture and washed in serum free media two times (Centrifugation at 1000rmp for 10 minutes). Cells were counted, assessing viability, and resuspended at 5 x 10^6/ml and 100µl aliquots were seeded onto 96 U well plates in triplicate. Cells were titrated to give a range of effector target ratios (E:T): 100:1, 50:1, 25:1 and 12:1 (5000 targets per well). Prepared targets were then added in co-culture with the CTL effectors at 5000 cell per well. 200µl total assay volume was used with complete media and in order to determine spontaneous and maximal lysis, wells without CTL effectors were used in triplicate either containing target cells alone or in conjunction with 20µl of triton X-100 detergent. Plates were then incubated for 4 hours at 37°C with 5% CO_2. 50µl aliquots of the assay supernatants were transferred to 96 well lumaplates (Packard Bioscience, Groningen, The Netherlands) containing solid scintillant and the plates were allowed to air-dry overnight at room temperature. The plates were then analysed on a Topcount scintillation counter (Canberra Packard, Pangbourne, UK). The results were analysed and the relative percentage cytotoxicity was calculated using the following formula: [(peptide specific ^{51}Cr release form CTL effectors – spontaneous ^{51}Cr release) / (maximum ^{51}Cr release – spontaneous ^{51}Cr release)] x 100.

2.7.5 Enzyme linked Immunospot assay (ELISpot)

This assay was used to determine the frequency of epitope specific responses generated by the DNA vaccines used. Analysis was performed for both IFNγ and IL4 specific production in response to *In Vitro* stimulation of immunised effectors with target peptides. The assay was performed using cytokine specific development modules and ELISpot blue colour modules from R & D Systems Europe Ltd (Abingdon, Oxon, UK) as per the manufacturer's guidelines. The total volume of required cytokine specific capture antibody was determined and diluted at a ratio of 1:60 in PBS. 100µl of the diluted antibody was then applied to each well of a 96 well PVDF membrane 'Immunospot' plate (multiscreen-IP, Millipore, Bedford, MA, USA), plates were covered and placed at 4°C overnight. The capture antibody was aspirated from the plate, which was then washed 4 times in sterile PBS containing 0.05% Tween 20. Care was taken to avoid damage to the plate's membrane. The

plates were blotted dry and the non specific binding sites were blocked with 200µl of sterile filtered 1% BSA in PBS, and incubated at room temperature for 2 hours. The blocking buffer was aspirated from the wells which were washed 3 times in PBS Tween 20. The membrane was then equilibrated by the addition of 200µl of complete cell culture media and placed at 37°C until seeding of immunised splenocytes was required. The culture media was then aspirated and 1 x 10^6 splenocytes were seeded per well in 100µl of culture media. Splenocytes were then stimulated under varying conditions: 10µg/ml target peptide added per well in triplicate, negative controls were wells containing only cells and wells with media alone and positive controls were set up containing 1µg/ml phorbol 12-myristate 13 acetate (PMA) and 200nM ionomycin. All wells contain a total assay volume of 200µl culture media. For the determination of epitope specific effector frequency, titrations of both the number of seeded effector cells and the concentration of peptide added were varied. Plates were then incubated at 37°C in 5% CO_2 for at least 16 hours. Plates are loosely wrapped in foil to prevent the development of diffuse spots. The splenocytes were aspirated from the plates and all wells were washed three times in PBS Tween. The total volume of cytokine specific detection antibody was calculated and diluted at a 1:60 ratio in sterile 1% BSA in PBS (sterile 0.2micron filtered). 100µl of the detection antibody was added per well and the plates were incubated at 4°C for at least 16 hours. The plates were then washed a further 4 times in PBS Tween, and blotted dry. 100µl of alkaline phosphatase conjugated streptavidin, diluted 1:60 in 1%BSA, was added per well and plates were incubated for 2 hours at room temperature. Plates were then washed five times in PBS Tween and once in distilled water. 100µl of the substrate, 5-broomo-4-chloro-3'indolylphosphate p-toludine salt (BCIP) and nitro blue tetrazolium chloride (NBT) solution was added per well and the plate was incubated at room temperature, in the dark, for 30 minutes. The BCIP/NBT solution was then aspirated from the plates which were washed three times in distilled water and allowed to air dry at room temperature. The plates were analysed using the Bioreader 3000 Pro (Camlab, Cambridge, UK).

2.8: Antibodies used throughout all experiments including concentrations and mode of action.

Direct Primary

Antigen	Fluorescent conjugate	Species of origin	Dilution /Conentration	Volume (µl) per100,000 cells	Supplier and address
C3b (C3c polyclonal)	FITC	Rabbit	1/100	100	DAKO A/5, Denmark

Indirect Primary

Antigen and Isotype	Species of origin	Dilution /Concentration	Volume (µl) per100,000 cells	Supplier and address
CD55 SCR 1 [(BRIC 220) (IgG_1)]	Mouse	5-10µg/ml	100	IGBRL, Bristol, UK
CD55 SCR 3 [(BRIC 216) (IgG_1)]	Mouse	5-10µg/ml	100	IGBRL, Bristol, UK
CD55 SCR 2 [(BRIC 110) (IgG_1)]	Mouse	5-10µg/ml	100	IGBRL, Bristol, UK
CD55 SCR 1-2 [(791T/36) (IgG_{2b})]	Mouse	5-10µg/ml	100	Purified in laboratory from hybridoma
CD55 SCR 1 [(IA10) (IgG_{2a})]	Mouse	5-10µg/ml	100	BD Pharmingen, Belgium
Control Ab (IgG)	Human	5-10µg/ml	100	Sigma, Missouri, USA
Human IgG (Fc specific)	Goat	5-10µg/ml	100	Sigma, Missouri, USA
Bovine Serum Albumin (IgG polyclonal)	Rabbit1	1/1000	100	Abcam, Cambridge, UK
CD55 [(IC6) (SCR3)]	Mouse	5-10µg/ml	100	GmbH, Germany
CD55 [(IH4) (SCR3)]	Mouse	5-10µg/ml	100	Dr B. Loveland
CD55 [(IA10) (SCR3) (IgG_{2a})	Mouse	5-10µg/ml & Titration	N/A	BD PharMingen, Belgium
HLA-A2 (BB7.2)	Mouse	5-10µg/ml	100	Purified in laboratory from hybridoma (ATCC Rockville, MD, USA)
365 (708) ($IgG2_b$)	Mouse	5-10µg/lm	100	Purified in laboratory from hybridoma

Secondary

Antigen	Fluorescent conjugate	Species of origin	Dilution /Concentration	Volume (µl) per100,000 cells	Supplier and address
Anti-mouse IgG	FITC	Goat	1/100	100	DAKO A/5, Denmark
Anti-mouse IgG	Hrp	Goat	1/1000	100	DAKO A/5, Denmark
Mouse CD19 (IgG2a)	FITC	Rat	1/1000	100	DAKO A/5, Denmark
Mouse IgG1	Hrp	Goat	1/1000	N/A	Serotec,Oxford,UK
Mouse IgG2a	Hrp	Goat	1/1000	N/A	Serotec,Oxford,UK
Human IgG (Fc specific)	Hrp	Goat	5-10µg/ml	100	Sigma,Missouri,USA

IGBRL: International Blood Group Reference Laboratory

Chapter 3: Development of monoclonal antibodies capable neutralising CD55

3.1: Introduction

Decay accelerating factor is highly expressed on many tumour cells, offering protection from autologous, complement bystander attack. The presence of CD55 on stromal cells surrounding tumours and the presence of soluble DAF would suggest that its expression is an important cofactor for tumour development.

Mutations in SCR 3 of CD55 have been shown to produce the most disruptive effects on its complement regulatory activity, when compared to mutations in other domains (Coyne et. al. 1992). An array of antibodies to specific domains within decay accelerating factor (CD55) were tested for their ability to block its regulatory activity, and only two antibodies, specific to SCR 3, completely abolished its function.

Blok et. al. (1997), showed that a bispecific antibody recognising CD55 and tumour cell antigen G250 induced C3 deposition and tumour cell lysis, and that opsonised cells became sensitised to complement mediated lysis. Similarly Zhong et. al. (1995) showed that increased tumour killing could be achieved by CD55 blockade with an antibody to SCR3 of CD55.

The preliminary aim was to generate monoclonal antibodies specifically targeting the SCR3 domain of decay accelerating factor, capable of neutralising its complement regulatory activity. This would lead to enhanced C3 deposition on the surface of tumour cells and ultimately produce elevated levels of tumour cell lysis and ultimately regression.

In order to generate anti SCR3 monoclonal antibodies, a relevant immunogen was required. Potentially immunogenic regions were identified with the use of hydrophobicity plots and synthesised. Utilisation of peptides as immunogenic targets enables generation of a specific response to a single epitope and therefore a peptide immunisation strategy was chosen.

Sera from immunised mice was collected and CD55 specific responses were assessed via ELISA, FACS binding and C3b deposition assays. Positive responders were

immortalised in order to generate monoclonal antibodies to be assessed for CD55 neutralising capabilities.

Results

3.2: Antigen prediction for generation of peptides

The peptide sequence for SCR3 domain of human CD55 was obtained via SWISS-PROT software (Accession number P90174). The sequence was entered into the ProtScale program in order to generate a hydrophobicity plot, illustrating regions within the peptide sequence that show hydrophilic characteristics. These regions indicate potential antigenic sites, which are likely to be exposed on the complete folded protein surface. The Kyte-Doolitle scale is widely applied for delineating hydrophobic character of a protein.

Figure 3.1 shows the individual values associated with all the amino acids present in the target sequence as generated by this plot scale. All regions within the sequence are given charge values relative to their position and adjacent amino acids. Sequences with values greater than zero are said to be hydrophobic in nature, while those with values less than zero are said to show hydrophilic properties and thus likely to be exposed as antigenic targets for antibody development.

Figure 3.1: Protein Hydrophobicity Plot of SCR3 domain of CD55 for peptide design.

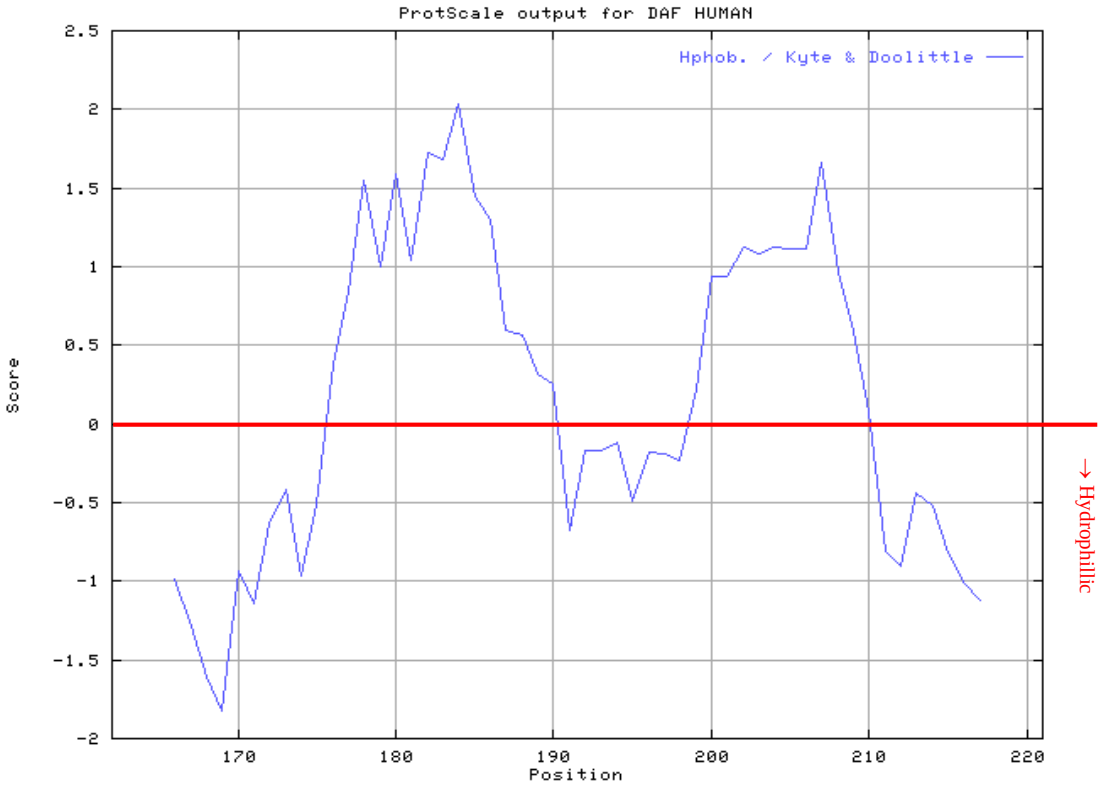
SCR3 of CD55 peptide sequence:
^{162}KSCPNPGEIR NGQIDVPGGI LFGATISFSC NTGYKLFGST SSFCLISGSS VQWSDPLPEC R^{221}

The peptide sequence of human Decay accelerating factor (accession #: P08174) was obtained via SWISS-PROT software. The sequence of SCR3 domain was entered into the ProtScale program in order to generate the Hydrophobicity plot: -

Regions with values greater than zero are hydrophobic in character.
Regions with values less than zero are hydrophilic and likely to be exposed on the surface of a folded protein.

Kyte & Doolitle Scale used.
The individual values for the 20 amino acids are:

Ala : 1.8	Arg : -4.5	Asn: -3.5	Asp: -3.5	Cys: 2.5	Gln: -3.500
Glu : -3.	Gly : -0.4	His: -3.2	Ile: 4.5	Leu: 3.8	Lys: -3.900
Met : 1.9	Phe: 2.8	Pro: -1.6	Ser: -0.8	Thr: -0.7	Trp: -0.900
Tyr : -1.3	Val : 4.2	Asx: -3.5	Glx: -3.5	Xaa: -0.49	



The three highlighted regions were selected as potential antigenic targets for antibody development. All peptides were unique to human CD55 when used in a BLAST search.

The sequence encoding the SCR3 of CD55 was aligned with SCR2 of CD46 to determine if homology would enable prediction of molecular structure (**Figure 3.2**). By comparing the crystal structure of CD46 with the SCR 3 domain of CD55 it was possible to produce a 3 dimensional model (**Figure 3.3**) confirming the surface locations of the chosen target peptides.

Figure 3.2: Alignment of CD55 SCR3 with CD46 SCR2

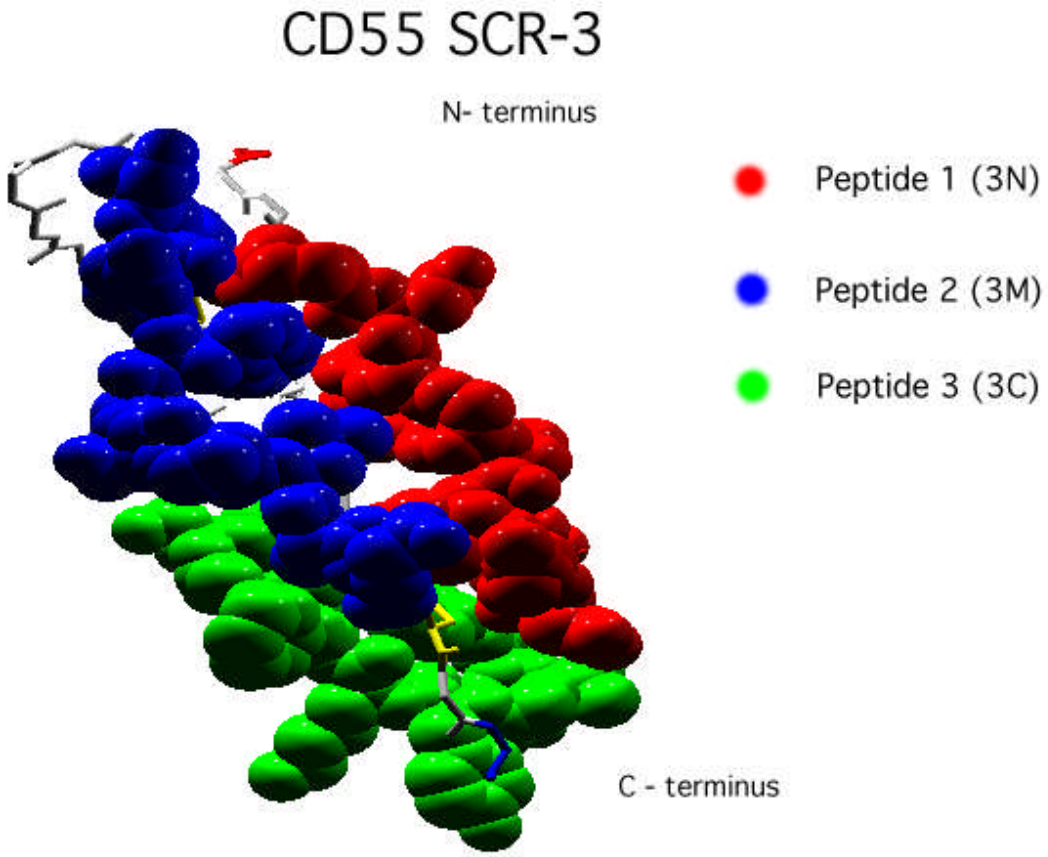
```
CD46 1-2    1   CEEPPTFEAM ELIGKPKPYY EIGERVDYKC KKGYFYIPPL ATHTICDRNH


CD55 3      1                KSCPNPGE IRNGQ-IDVP GGILFGATIS FSCNTGYKLF
CD46 1-2   51   TWLPVSDDAC YRETCPYIRD PLNGQAVPAN GTYEFGYQMH FICNEGYYLI
                           . .**    .    *** .     *    **   .   * ** ** *.

CD55 3     39   GSTSSFCLIS GSSVQWSDPL PECRE
CD46 1-2  101   GEEILYCELK GSVAIWSGKP PICEKV
                         . **.. **     *
```

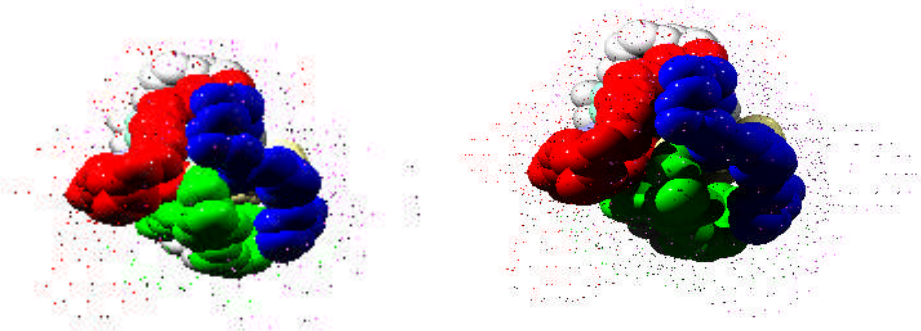
The residues marked in red are highly conserved between all SCR domains.

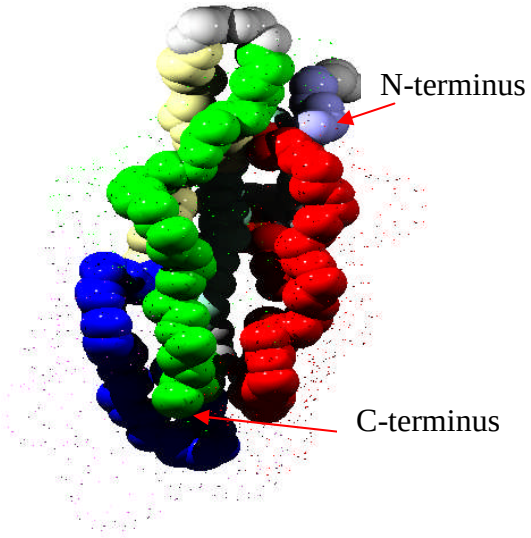
Figure 3.3: Prediction of 3 dimensional model of target peptides, generated by comparison with crystal structure of CD46 short consensus repeat domains.



Representation of three loops of peptides predicted to be hydrophilic, occupying surface locations on CD55.

The presence of four conserved cysteins, constrain the structure and conserved bulky hydrophobic residues occupy the interior of the model. This leaves loops of protein (target peptides) at the surface. Below is a view of the structure from beneath showing the predicted surface locations of the chosen peptides. The dots surrounding the image represent predicted surface shape occupied by hydrogen and water molecules.





Hoppe-Woods (1980) & Janin (1978)

Three regions within the sequence were identified as potential surface epitopes for peptide generation and immunisation:

CD55 3N	$^{N'}$CPNPGEIRNGQIDVPG$^{C'}$	16mer	N terminal peptide
CD55 3M	$^{N'}$CNTGYKLFGST$^{C'}$	11mer	Middle peptide
CD55 3C	$^{N'}$SVQWSDPLPEC$^{C'}$	11mer	C terminal peptide

The three peptide sequences were used in a BLAST search and all were shown to be unique, indicating that antibodies generated would be specific to these regions located within complete CD55. As well as being chosen for predicted surface location and hydrophobicity, the peptides were selected to include a cystein residue, which can be used to attach the peptides to a carrier protein, such as KLH. The peptides were synthesised by the BSAU at Nottingham using Fmoc chemistry assessing purity by HPLC and mass spectrometry .

3.3: Immunisation Protocol

Three groups of three mice (N, M and C) were immunised with target peptides at two weekly intervals, table C shows the immunisation protocol followed and the subsequent analysis of tail bleeds and cell fusions carried out.

Table 3.1: Immunisation/Screen protocol.

Week	Immunisation / Immunogen	Bleed	Screen
1	1°: 100µg KLH conjugated peptide + CFA s.c.	N/A	N/A
3	2°: 100µg KLH conjugated peptide + IFA i.p.	N/A	N/A
5	3°: 100µg KLH conjugated peptide + IFA i.p.	**1°**	OVA-peptide (ELISA) -peptide (ELISA)
7	4°: 10^5 CHO HI DAF cells + IFA i.p.	**2°**	BSA-peptide (ELISA Native CD55 (791T)cells (ELISA) Purified CD55 (ELISA)
9	5°: 100µg KLH conjugated peptide + IFA i.p.	**3°**	BSA-peptide (ELISA)
11	6°: 100µg KLH conjugated peptide + IFA i.v.	N/A	N/A
12	Collect splenocytes and fuse with NSO	N/A	Hybridoma screens against peptide/ purified CD55 / native antigen/Complement deposition

Summary of table definitions:

KLH : Keyhole Limpet Haemocyanin (peptide carrier)

OVA : Ovalbumin (peptide carrier)

IFA : Incomplete Freund's adjuvant

CHO HI DAF : Chinese Hamster Ovary cells transfected to express CD55

N/A : Not Applicable

Combinations of screening protocols were developed in order to assess specificity of generated antibodies. An Elisa screen was utilised to determine if recognition of primary target peptide coupled to different carrier proteins could be observed and FACS analysis was utilised to confirm whether antibodies recognised complete native antigen as expressed on cells. Hybridomas were also screened via Elisa against target peptides and purified CD55, again analysing whether the antibodies generated could recognise native antigen formation. Finally hybridoma supernatants were assessed via a complement deposition assay to determine if blocking of CD55 function could be produced.

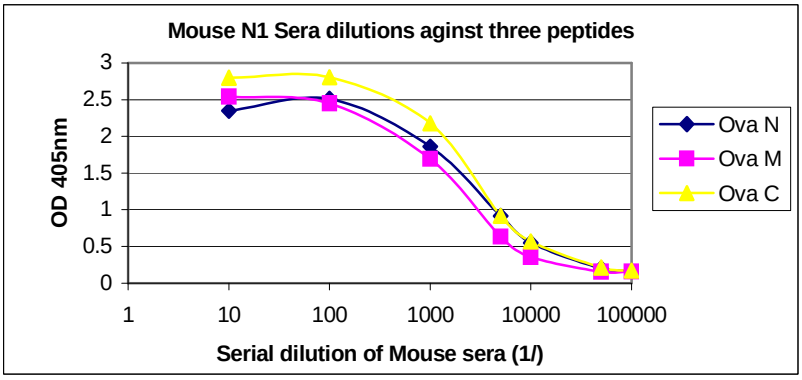
3.4: Screening of mouse sera for presence of target specific antibodies

Three groups of mice were immunised with 100µg KLH conjugated peptides (N, M and C) with Complete and Incomplete Freund's Adjuvant sub-cutaneously and intra-peritonealy; tail bleeds were obtained 5 days post immunisation. Erythrocytes were removed via centrifugation and the sera were collected, titrated and screened via ELISA against target peptides (as **Table 3.1**).

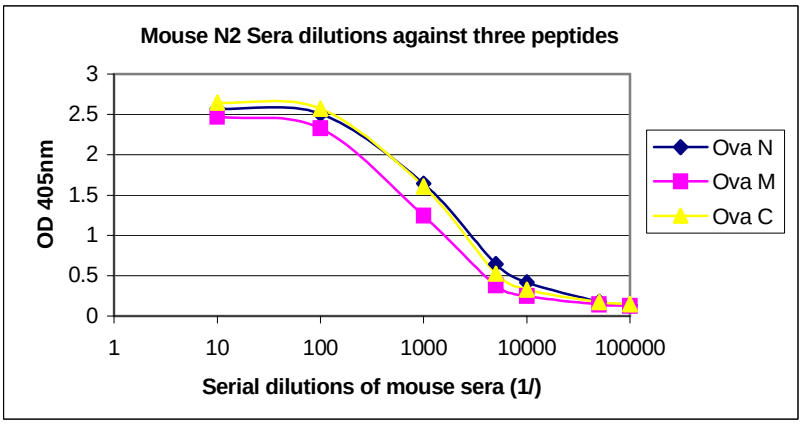
96 well Elisa plates were coated with ovalbumin conjugated N, M and C peptides and non-specific binding sites were blocked with BSA. Titrated mouse sera was then added in triplicate and binding was assessed using secondary HRP conjugated antibodies and the ABTS detection system.

Figure 3.4 represents the results obtained from the primary bleeds, sera from all mice screened against all three ovalbumin-conjugated peptides. All three groups show that strong primary responses have been generated to specific peptides and the current results showing recognition at dilutions of 1 in 5000. However all mice sera also show strong recognition to alternative peptides, indicating a degree of non-peptide specific binding. This may be due to the presence of ovalbumin conjugated to the target peptides, which may be 'sticky' due to its innate charge and structure. N peptide immunised mice show predominantly stronger binding to the N peptide targets, and both M and C immunised mice show strongest responses to their relative peptides. A titre of 1 in 10,000 indicates significant response levels for future fusions and antibody development.

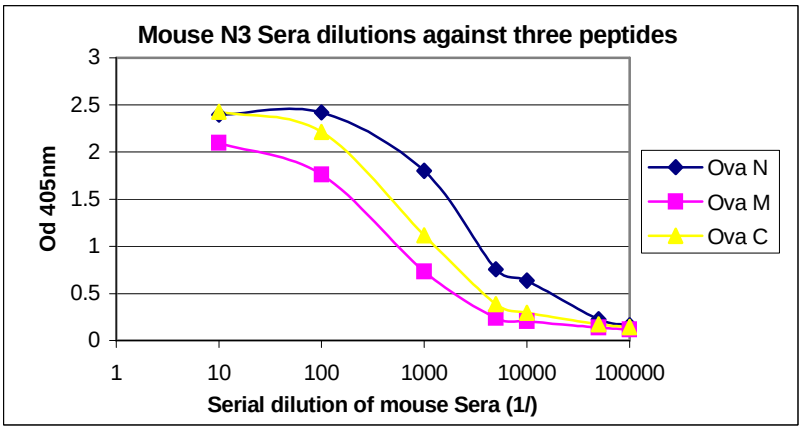
Figure 3.4: Mouse sera titration for presence of peptide specific antibody.
Three groups of mice immunised with KLH conjugated peptides (N, M and C). 5µg/ml of ovalbumin-conjugated peptide coated plates overnight at 4°C. Primary and secondary antibodies were added before using the ABTS system for ELISA detection. Error bars covered by symbols and plates read at 405nm. Sera from each mouse analysed against all three synthesised peptides.



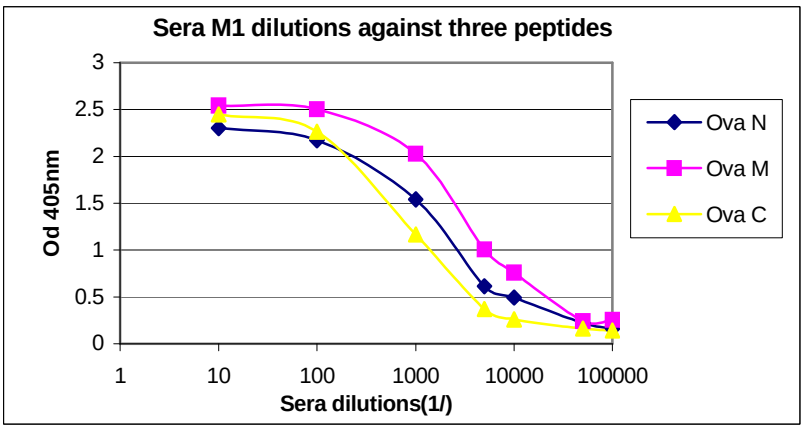
Mouse N1: Immunised with N-KLH peptide.



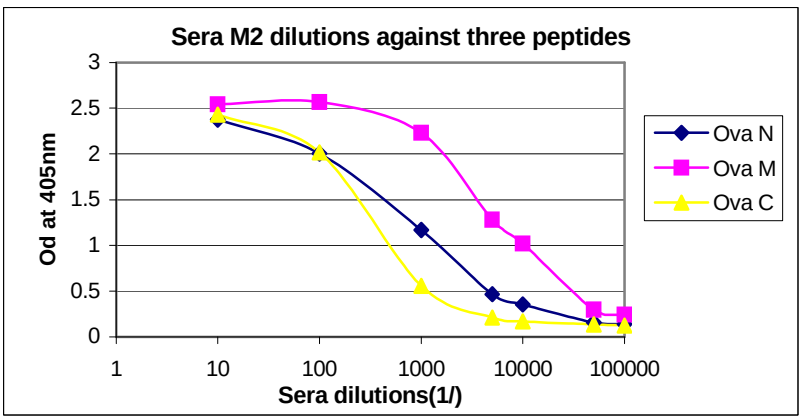
Mouse N2: Immunised with N-KLH peptide.



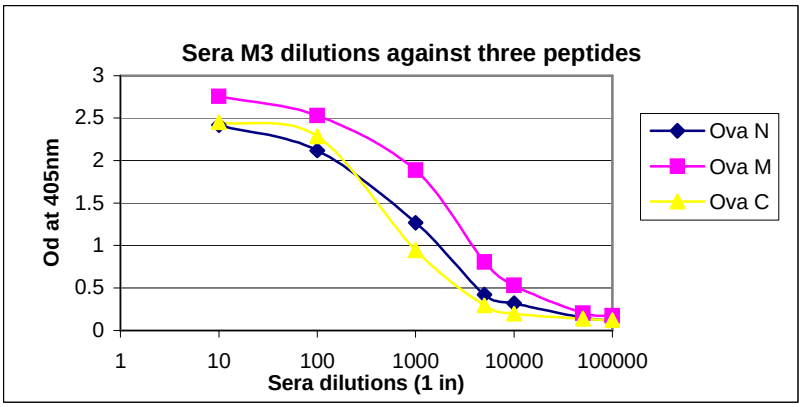
Mouse N3: Immunised with N-KLH peptide.



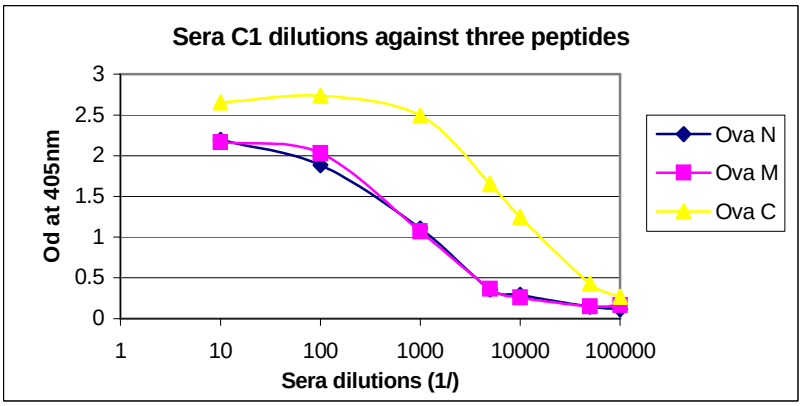
Mouse M1: Immunised with M-KLH peptide.



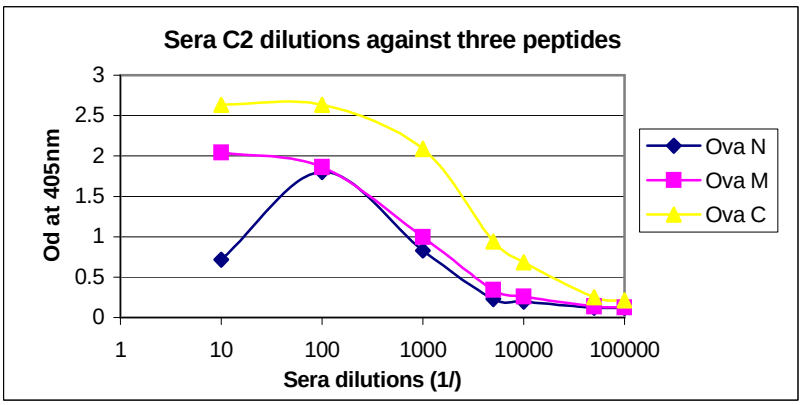
Mouse M2: Immunised with M-KLH peptide.



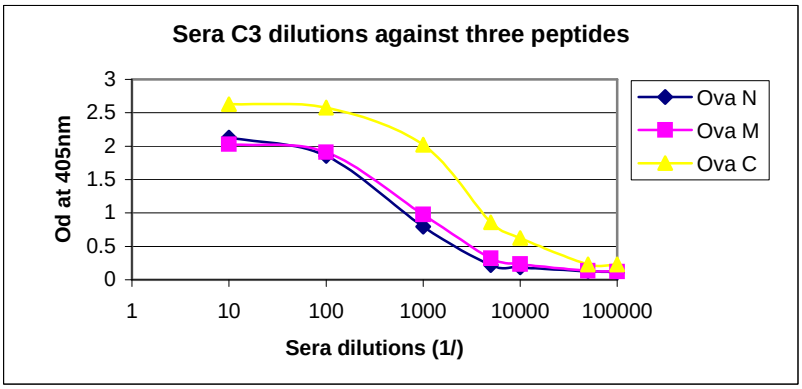
Mouse M3: Immunised with M-KLH peptide.



Mouse C1: Immunised with C-KLH peptide.



Mouse C2: Immunised with C-KLH peptide.

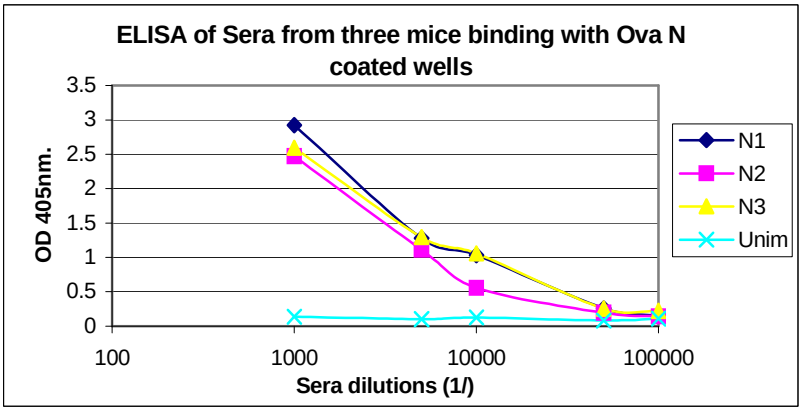


Mouse C3: Immunised with C-KLH peptide.

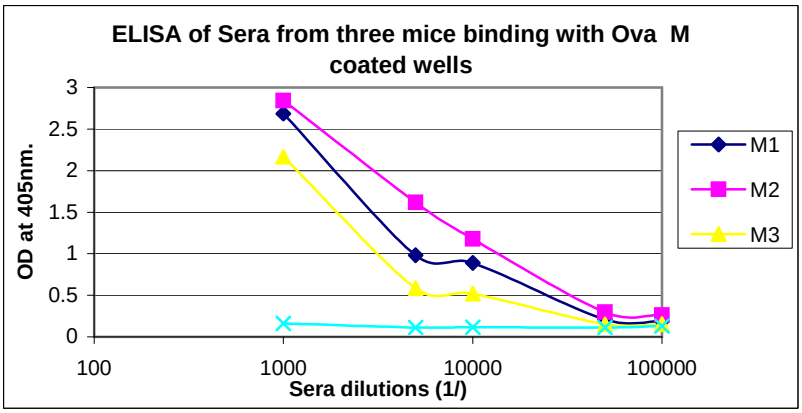
Group immunisations were compared in order to assess which mice were generating the greatest responses to their target peptides (**Figure 3.5**) when compared to serum from an un-immunised individual. All three groups show strong peptide recognition at dilutions up to 1 in 10000 with significant difference compared to un-immunised sera. Mice N1, M2 and C1 currently show the greatest level of response to their target peptides.

Figure 3.5: Mouse Sera titration for presence of peptide specific antibody. Sera screened against target peptide, compared to sera from an un-immunised mouse.

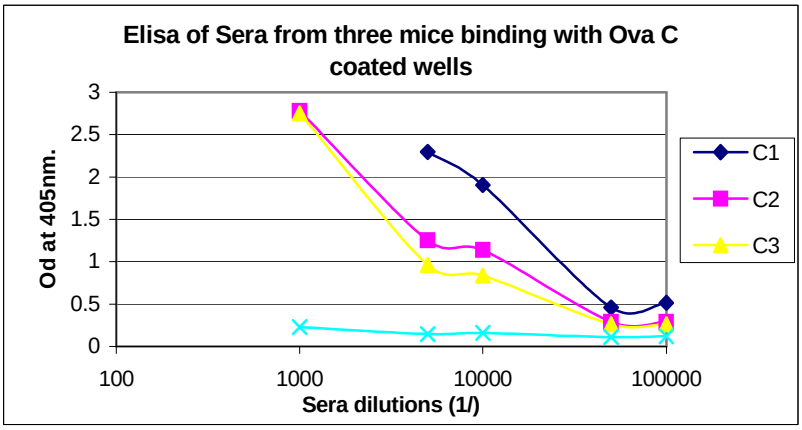
Plates coated with 5µg/ml ovalbumin-conjugated peptide overnight at 4°. Primary and secondary antibodies added before using the ABTS system for ELISA detection. Plates read at 405nm and error bars are covered by symbols.



N Group: Immunised with M-KLH peptide.



M Group: Immunised with M-KLH peptide.

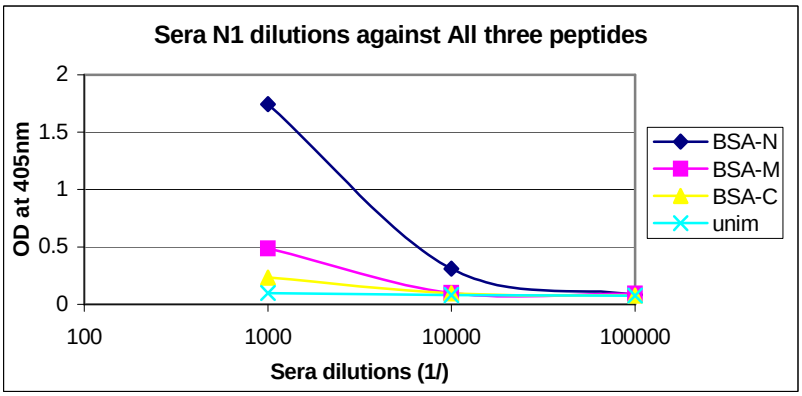


C Group: Immunised with C-KLH peptide.

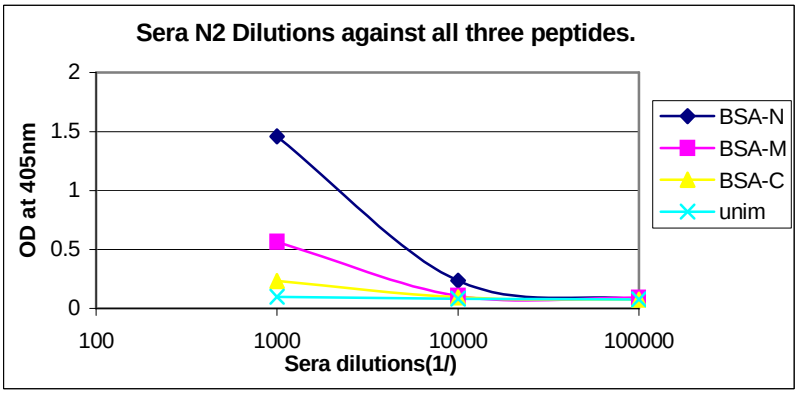
As sera from all mice show responsiveness to all ovalbumin-conjugated peptides, new targets were generated linking N, M and C peptides to BSA. Ovalbumin is a relatively large, charged molecule, which may induce interactions with antibodies in a non-specific manner. **Figure 3.6** shows primary bleed screens against new conjugated peptides when compared against un-immunised sera. Cross recognition of peptides is now reduced to results comparable with un-immunised serum, indicating removal of non-specific peptide recognition. All mice show strongest response to their specific peptide to a titre of 1 in 10000.

Figure 3.6: Mouse sera titration for presence of peptide specific antibody. Sera screened against new BSA-conjugated N, M and C peptides.

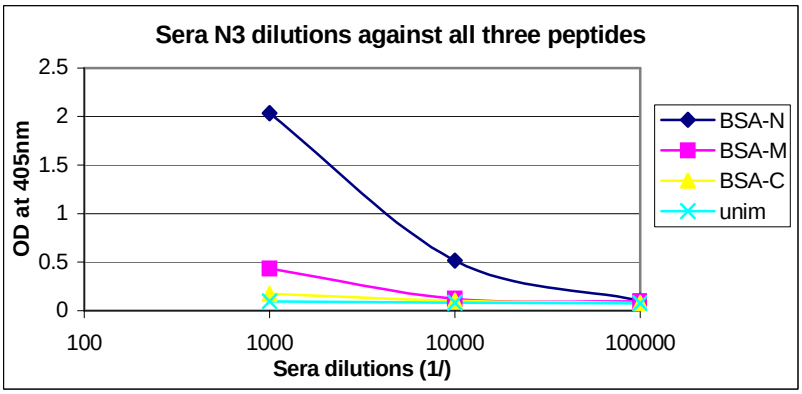
New peptide conjugates used to determine if previous cross binding was due to ovalbumin recognition. 5µg/ml BSA peptide coated plates at 4°C overnight. Primary and secondary antibodies were added before using ABTS system for ELISA detection. Plates read at 405nm and standard deviation covered by symbols. Sera from each mouse were analysed against all three BSA-conjugated peptides.



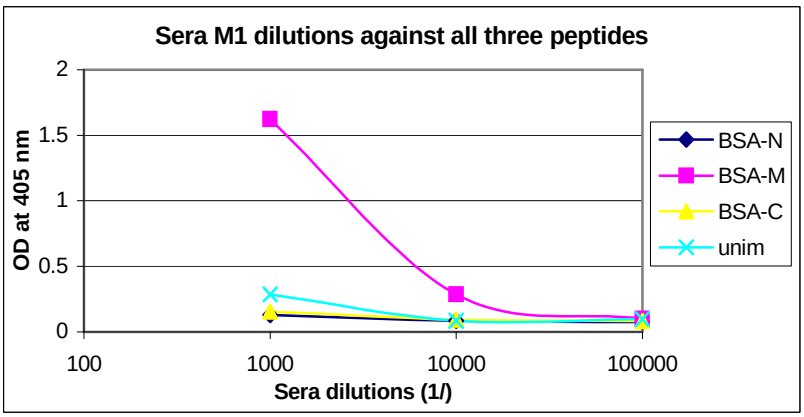
Mouse N1: Immunised with N-KLH peptide.



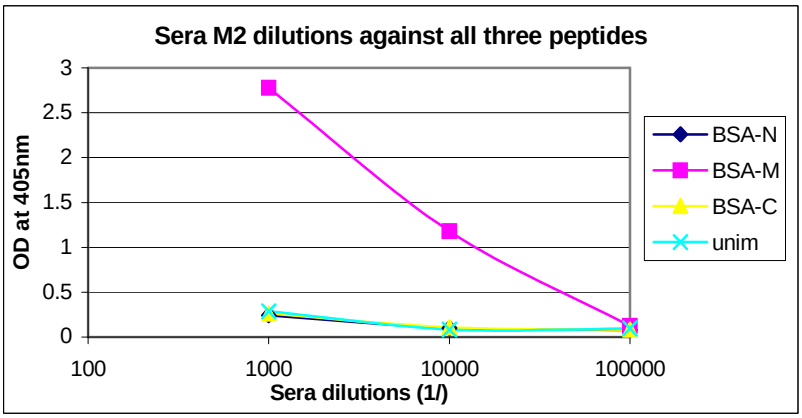
Mouse N2: Immunised with N-KLH peptide.



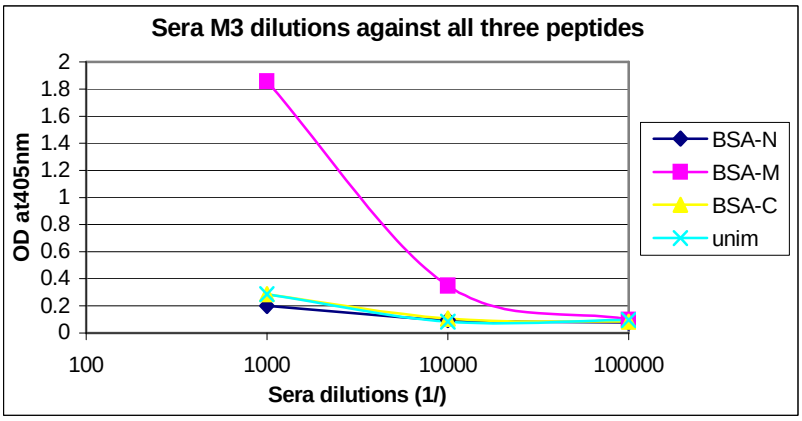
Mouse N3: Immunised with N-KLH peptide.



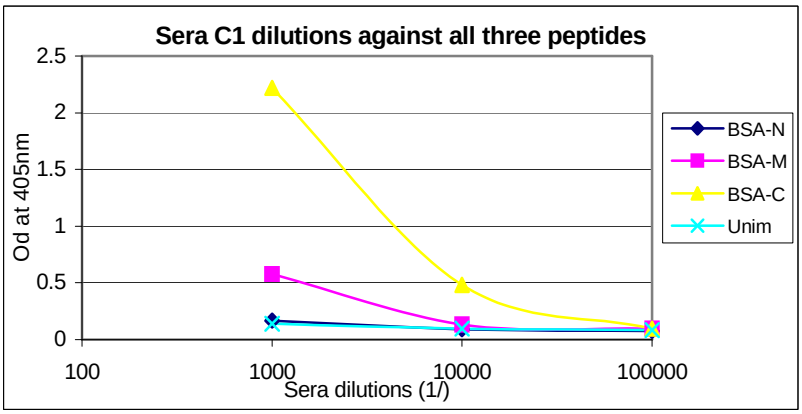
Mouse M1: Immunised with M-KLH peptide.



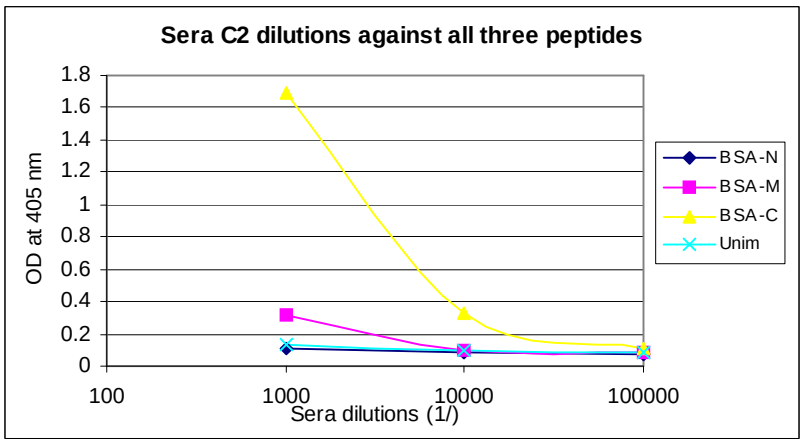
Mouse M2: Immunised with M-KLH peptide.



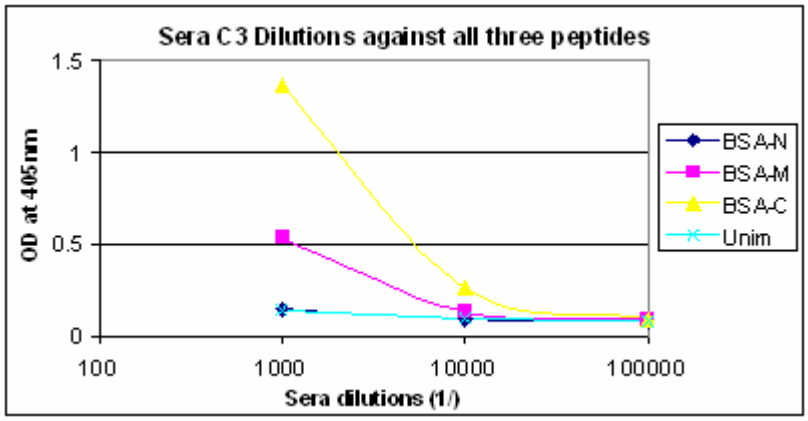
Mouse M3: Immunised with M-KLH peptide.



Mouse C1: Immunised with C-KLH peptide.



Mouse C2: Immunised with C-KLH peptide.



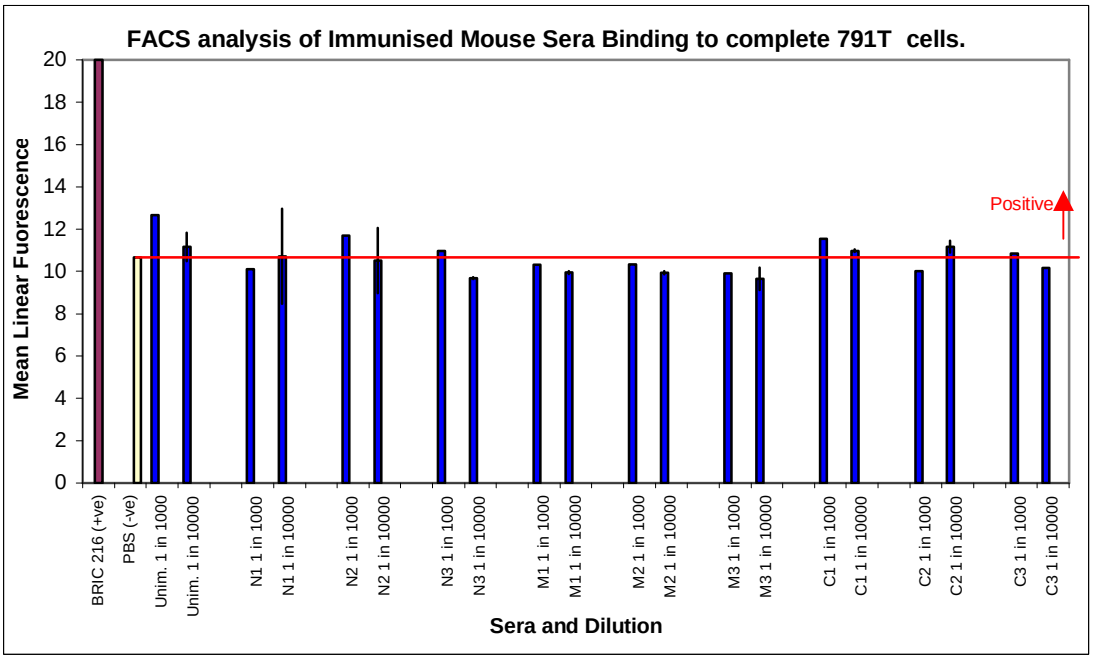
Mouse C3: Immunised with C-KLH peptide.

As anti-serum has now been shown to recognise target peptides conjugated to alternative carriers, it can be proposed that antibody responses have been generated to the target epitope. To assess this, sera were screened against complete native conformational antigen (CD55), naturally expressed on the osteosarcoma cell line 791T. **Figure 3.7** represents primary bleed sera recognition of CD55. 5 x 10^5 791T cells (CD55 over expressing osteosarcoma line) were coated with 100µl of titrated sera and assessed by flow cytometric analysis on the FacScan. BRIC 216 (SCR3 specific) monoclonal antibody was used as a control indicating the presence of conformational CD55 and PBS was used as a negative control for secondary antibody alone-labelled cells. Sera were used at 1 in 1000 and 1 in 10000 dilutions, these being dictated by limited sera volumes, though several previous maximum responses were seen at 1/10000. Results indicate limited recognition of complete native antigen as compared to the PBS control.

Primary bleeds indicate that generated antibodies display strong binding to peptide, although these antibodies did not recognise conformational native CD55. In order to selectively expand a B cell population, which recognises both peptide and complete CD55, peptide immunised mice were boosted with Chinese Hamster Ovary (CHO) cells transfected with a vector expressing complete CD55. As CHO cells are of rodent origin, many expressed surface proteins may not be immunogenic within the mouse system. Therefore the most immunogenic region present on the CHO cells should be conformational CD55.

Figure 3.7: Indirect labelling of 791T cells with titrated serum to determine if antibodies generated to peptide recognise native antigen.

5 x 10^5 791T cells coated with 100µl titrated sera for 1 hour at 4°C. Secondary antibody was FITC-conjugated and cells were assessed by flow cytometric analysis. Error bars indicate the range of two data points. Controls used: BRIC 216 and PBS.



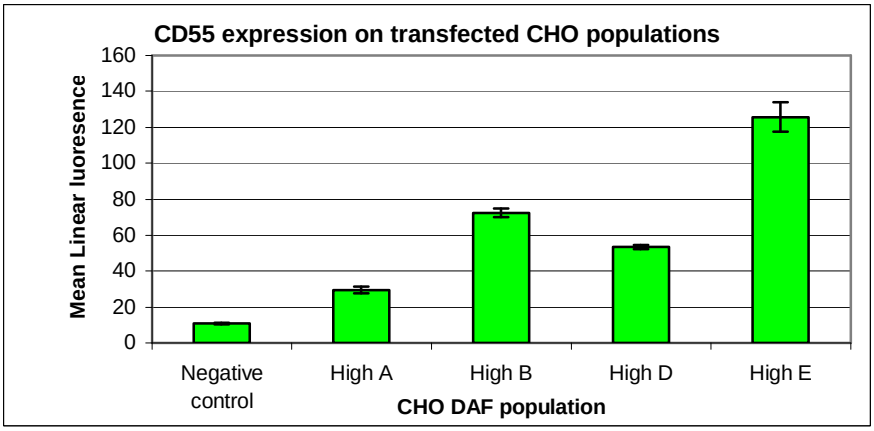
3.5: CD55 expression on stably transfected CHO cell line

CHO cells were transfected using the lipofectamine reagent method (*Invitrogen)* with **pApex** vector containing CD55 cDNA. Stable lines were generated with puromycin selection, and labelling of 5 x 10^5 transfected cells with 791T/36 (SCR1-2 specific) monoclonal antibody determined CD55 expression. Cells were incubated for 1 hour at 4°C with 791T/36 and washed with PBS. Labelled cells were assessed by flow cytometry. **Figure 3.8** shows four populations of transfected cells assayed when compared to un-transfected CHO cells. All populations were transfected under the same conditions using a range of DNA concentrations for the transfection protocol. The CHO A to E populations show a range of expression levels equating to 0.5, 1.0, 1.5 and 2.0µg DNA respectively. The CHO high E population showed the greatest level of CD55 expression and was used to immunise the peptide-primed mice.

Figure 3.8: Cytofluorimetric analysis of CD55 expression on transfected CHO cells.

5 x 10^5 cells labelled with 791T/36 antibody for 1 hour at 4°C. Cells analysed with FITC-conjugated Rabbit anti mouse antibody and read by flow cytometry. Transfected cells

populations compared to non-transfected CHO cells (negative control). All transfectants produced following identical protocol with varying DNA concentrations. Deviation expressed as two data points.



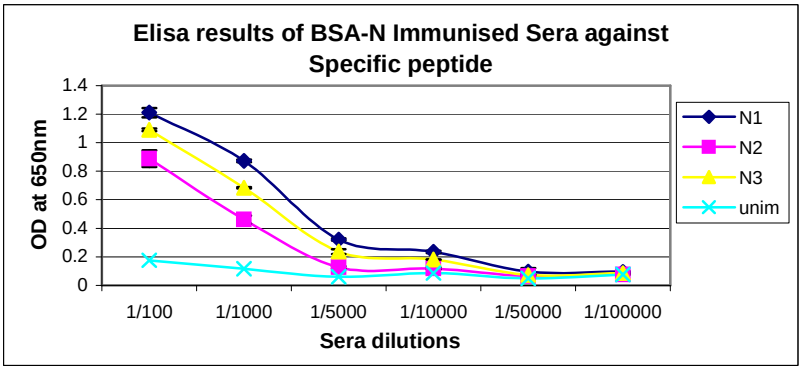
High A: 0.5µg DNA High B: 1.0µg DNA High D: 1.5µg DNA High E: 2.0µg DNA

3.6: Screen of secondary bleeds following immunisation with CHO High E cells.

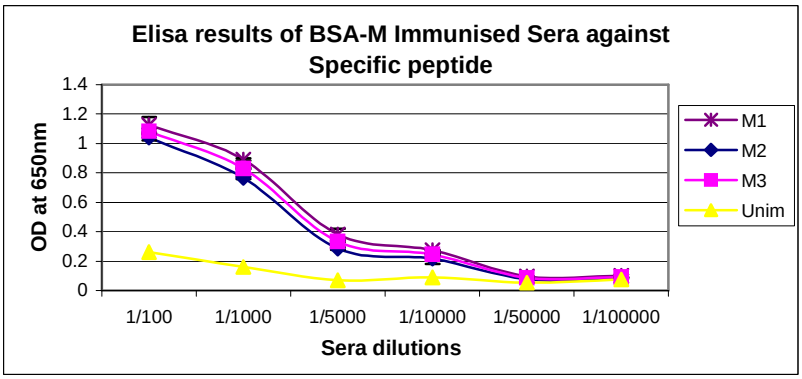
Mice have received two peptide immunisations and one boost with conformational CD55 on CHO-55 cells. **Figure 3.9** represents results of secondary bleeds screened via ELISA against BSA conjugated target peptides. Results show that responses to peptide are observed at sera titres of 1 in 10,000 when compared against un-immunised sera. These results are comparable to previous screen titres against peptide before cell boost. Mice N1, M1 and C1 show greatest responses to their target peptides when compared against all mice from within the same group.

Figure 3.9: Mouse sera titration of 2° bleeds for presence of peptide specific antibody.

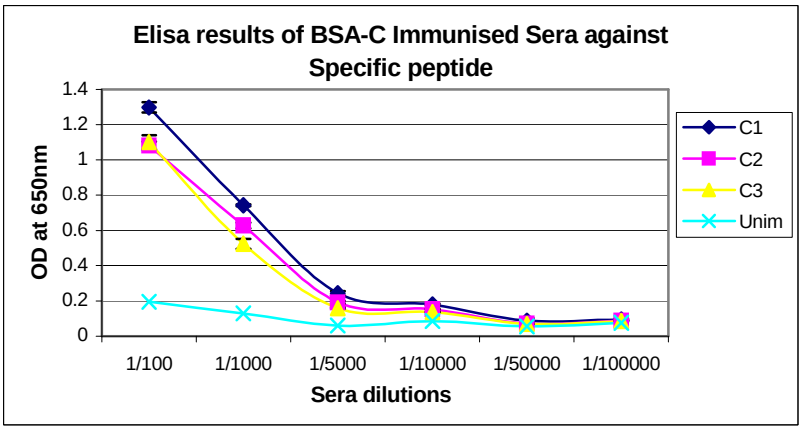
New bleeds collected following secondary immunisation with CHO DAF Hi E cells.5µg/ml of BSA conjugated peptides coated plates overnight at 4°C. Primary and secondary antibodies added before using the ABTS system for ELISA detection and read at 405nm. Sera screened against primary target peptides.



Mouse N1, N2 and N3 screened against N-BSA peptide.



Mouse M1, M2 and M3 screened against M-BSA peptide.



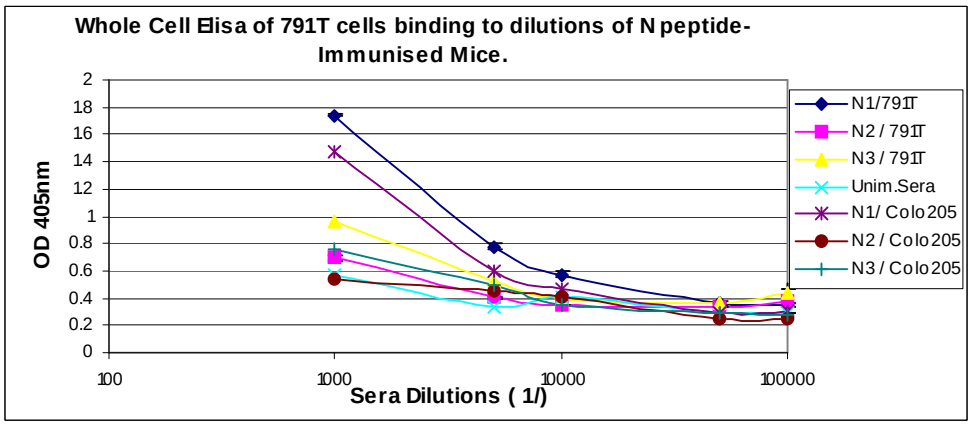
Mouse C1, C2 and C3 screened against C-BSA peptide.

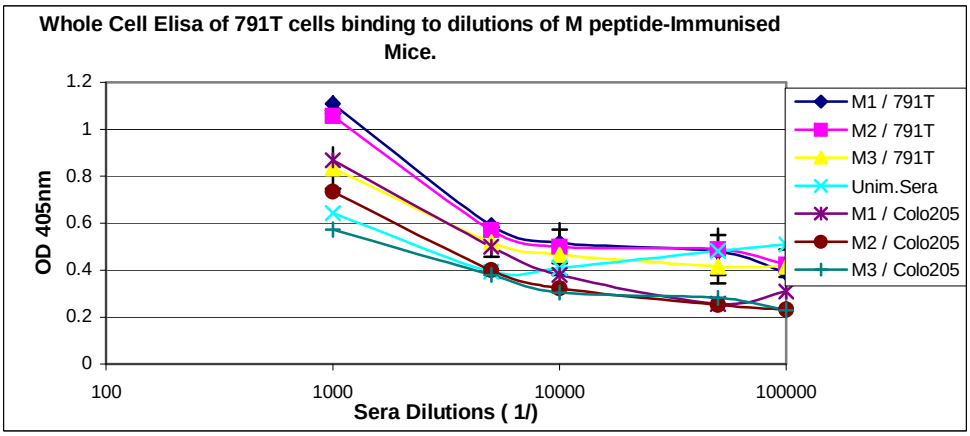
Sera were again screened to determine whether antibodies showed recognition of native antigen, which was achieved, by whole cell ELISA (**Figure 3.10**). Sera were assessed by indirect labelling of 791T (CD55 expressing) and Colo 205 (Non-CD55 expressing) cell lines. 5 x 10^4 cells were added per well on a 96 well ELISA plate and fixed with 0.5% gluteraldehyde. Non-specific sites were blocked and assessment was completed with ABTS reagent. Results indicate slight recognition of CD55 on 791T cells when compared to binding of Colo205 cells. All sera binding was compared to values generated with un-immunised sera. The responses were shown at dilutions of up to 1 in 5000 and sera from all groups show greater response to CD55 expressing cells compared to the CD55 negative cell line.

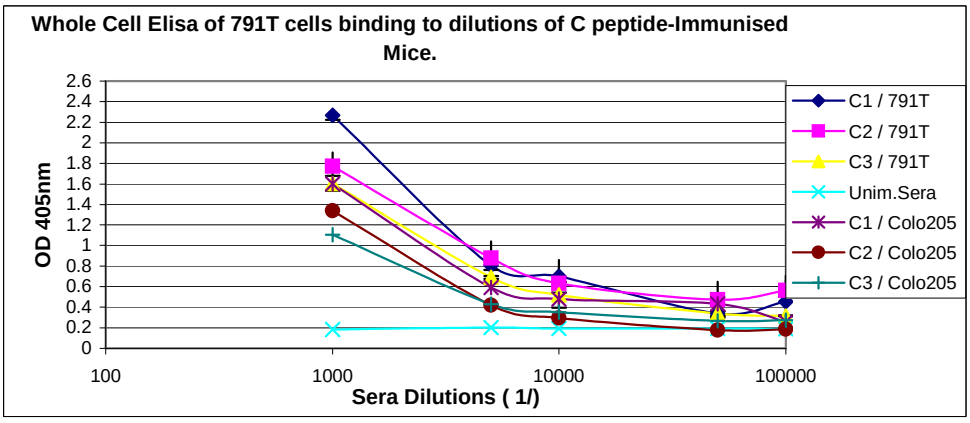
Figure 3.10: Indirect labelling of 791T and colo 205 cells with titrated 2° sera.

5 x 10^4 cells added per well in 96 well ELISA plate, incubated overnight at 37°C and fixed with 0.5% gluteraldehyde. Titrated mouse serum used as primary antibody, Rabbit anti mouse HRP conjugate used as secondary antibody prior to use of ABTS system for ELISA

detection. Plates read at 405nm and deviation shown for two data points (covered by symbols).





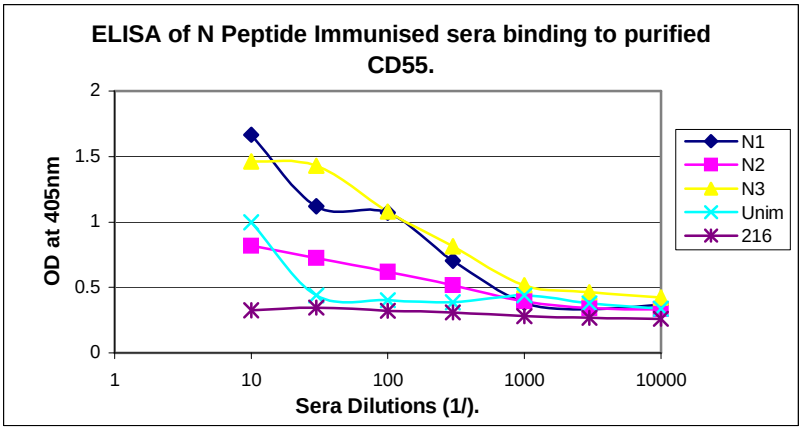


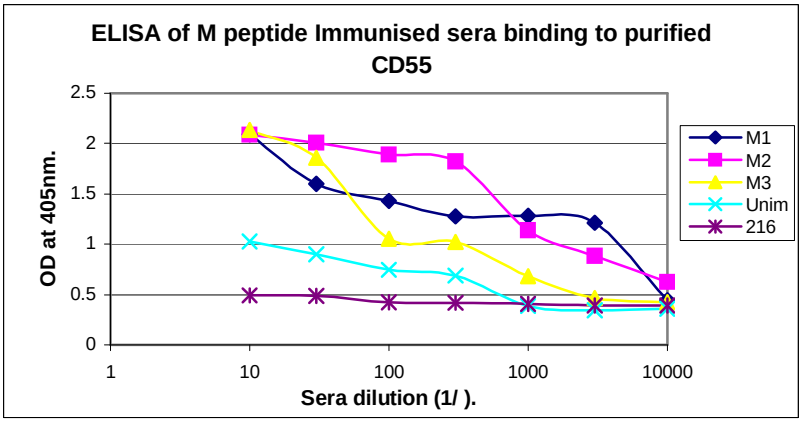
As sera screens show partial recognition of natively expressed CD55 via whole cell ELISA but not in the primary bleed FACS analysis, sera were screened against CD55 previously purified from erythrocytes. Analysis was completed via ELISA and 96 well plates were coated with 5µg/ml-purified CD55. Sera dilutions were added as primary antibodies and compared to un-immunised sera and BRIC 216 as controls

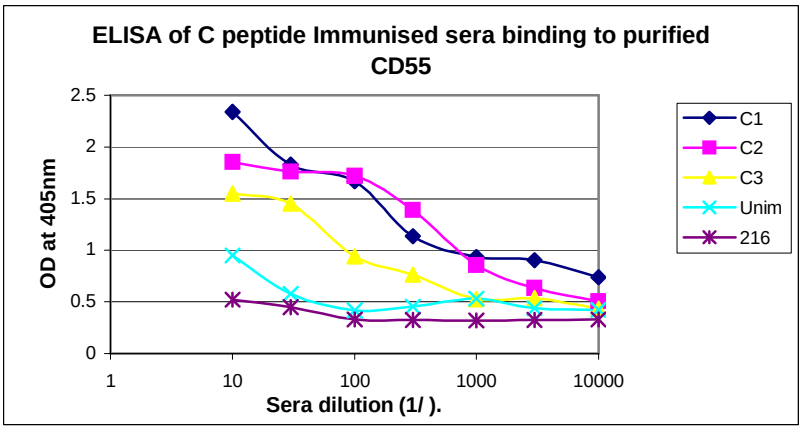
(**Figure 3.11**). All sera showed significant difference of recognition when compared to un-immunised serum for purified CD55. Both M and C immunisation groups showed responses to CD55 at titres if 1 in 5000, with M2 and C1 producing the strongest positives. However, BRIC216 showed no recognition for the purified CD55 indicating that upon binding to the ELISA plate, the conformational structure of protein may no longer be native. This finding may support previous results, indicating that generated antibodies recognise denatured and not conformational CD55.

Figure 3.11: Screen of 2° bleeds against purified CD55.

5µg/ml CD55 incubated overnight at 4°C. Primary titrated sera and secondary rabbit anti mouse HRP conjugate added prior to use of ABTS system for ELISA detection. Optical density read at 405nm and deviation showed as two data points (covered by symbols). Group immunisations screened against purified CD55.



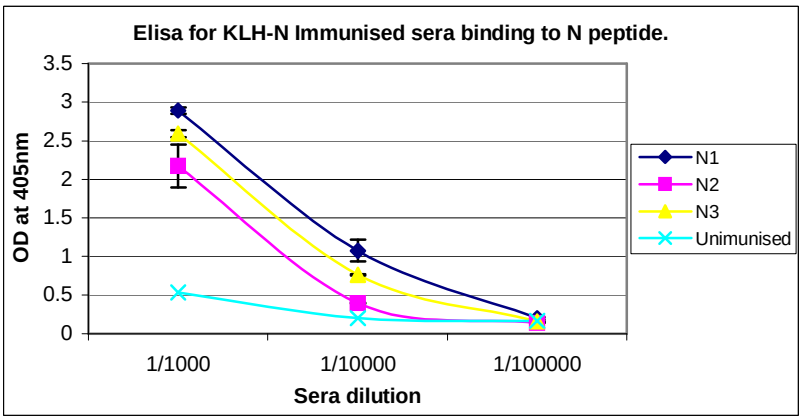


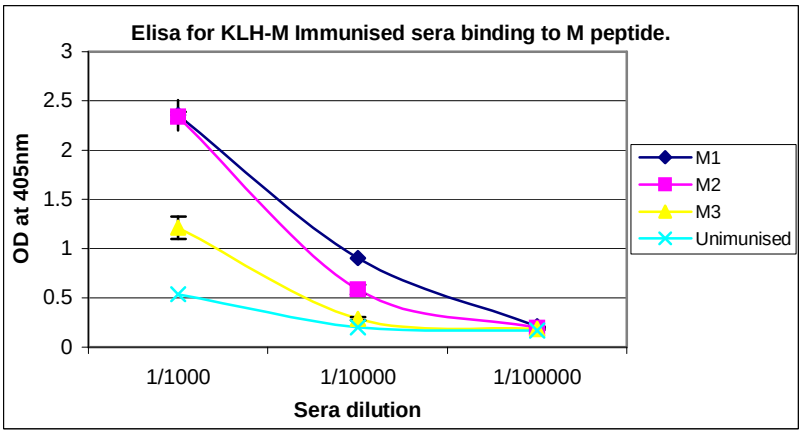


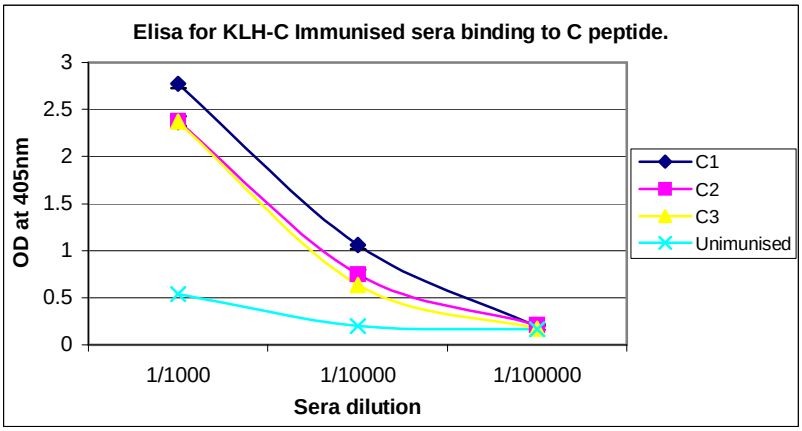
All mice were boosted with peptide conjugates to prevent driving of immune responses towards other antigenic regions on CD55 and 3° bleeds were screened via ELISA against BSA conjugated target peptides (**Figure 3.12**). All groups show significant response and titres of 1 in 10,000 and a significant difference is observed with immunised sera compared to un-immunised sera. Mice N1, M1 and C1 show the greatest response to peptide within their groups.

Figure 3.12: Mouse sera titration of 3° bleeds following target peptide boost for presence of peptide specific antibody

5µg/ml BSA-conjugated peptides coated on 96 well ELISA plate. Primary sera dilutions and secondary rabbit anti mouse-HRP conjugated antibody added before using the ABTS system for ELISA detection. Optical density read at 405nm and deviation shown for two data points (hidden by symbols). Sera screened against primary target peptides.







Due to previous strong positives with titres greater than 1/10000 against peptide and partial recognition against cells assessed by whole cell ELISA and purified CD55 ELISA, mice M1, M2 and C1 were immunised with 100µg KLH conjugated peptide intravenously as a final boost. As per protocol (**Table 3.1**), all three mice were sacrificed and splenocytes were collected. These cells were fused with the myeloma cell line NSO for hybridoma production.

3.7: Screening of hybridomas from M and C group fusions

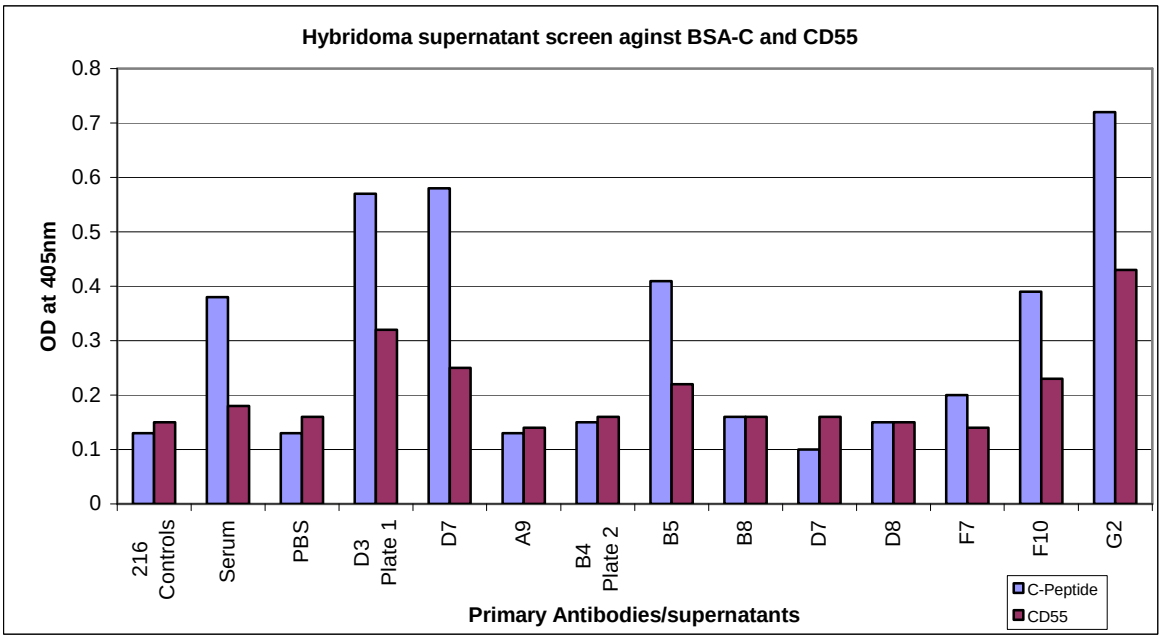
Mouse C1 and Mice M1 and M2 splenocytes were fused with NSO myeloma cells with the use of polyethylene glycol. Fusions were grown in HAT selective media over rat peritoneal exudates cells. Hybridomas were screened for target peptide recognition (ELISA), complete purified CD55 recognition (ELISA), specificity for native antigen expressed by osteosarcoma cell line 791T (FACS) and finally

inhibition of functional CD55 on 791T cells leading to increased complement deposition (FACS).

3.8: C hybridoma screens and development

Figure 3.13 represents results obtained for C-hybridomas screened against BSA-C peptide and purified CD55. Two plates of hybridomas were assayed and only wells containing viable cells were graphically represented. 5μg/ml peptide and CD55 were coated on 96 well plates and BRIC 216 and mouse serum (1/2000) were used as controls and added with 50μl hybridomas supernatant as primary antibody. ABTS substrate was used to assess antibody specificity. BRIC 216 does not recognise peptide sequence fractions from SCR3 or purified CD55, with results comparable to PBS negative control. All hybridomas show limited recognition of CD55 in comparison to peptide binding, suggesting that the produced antibodies recognise peptide with greater affinity than native CD55. Several clones show significant responses to peptide producing OD values up to 2.5 times background. CP1 D3 and CP2 G2 produce the greatest responses to both purified CD55 and BSA conjugated peptide. Both of these wells were cloned across 96 well plates at 0.3 cells per well.

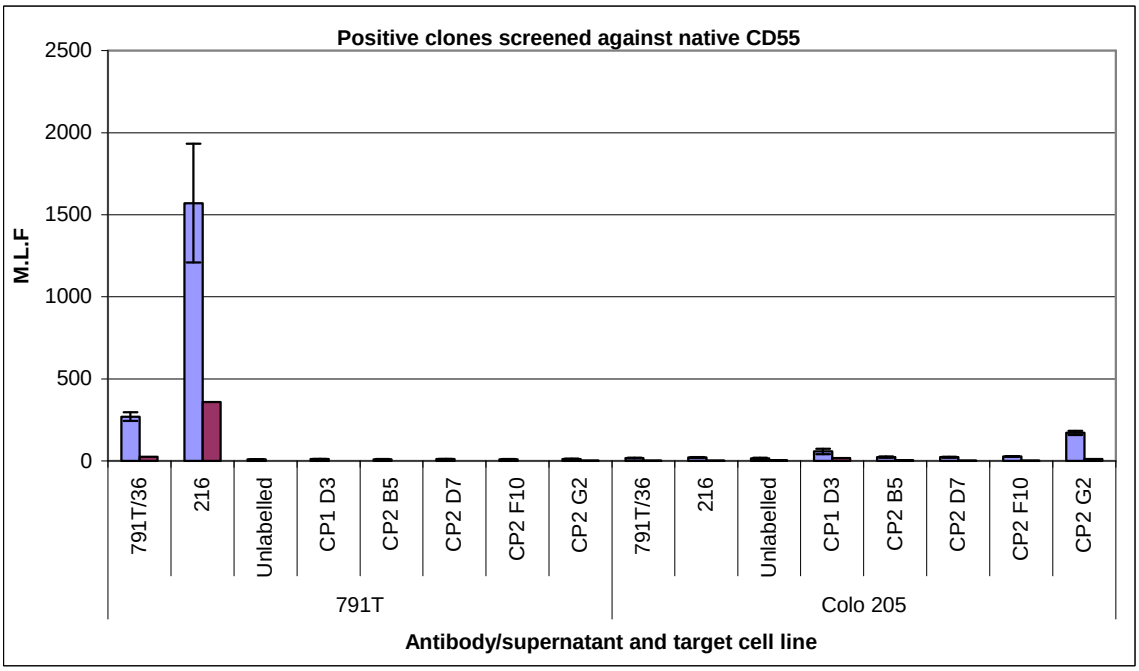
Figure 3.13: Screen of C1 hybridomas for recognition of target peptide and purified complete CD55.
Mouse C1 fused with NSO myeloma cells and grown in HAT selective media. Supernatants screened via ELISA for presence of peptide specific antibodies. 5µg/ml BSA- conjugated C peptide and purified CD55 were coated on 96 well plates overnight at 4ºC. 50µl of hybridoma supernatant was then added as primary antibody and secondary rabbit anti-mouse HRP conjugate was added and incubated for 1 hour prior to ABTS substrate addition. Plates were read at 405nm. Only data from wells containing viable cells shown.



CP1 and **CP2**: C peptide, plates 1 and 2.
CP1 D3 and CP2 G2 cloned 0.3 cells per well.

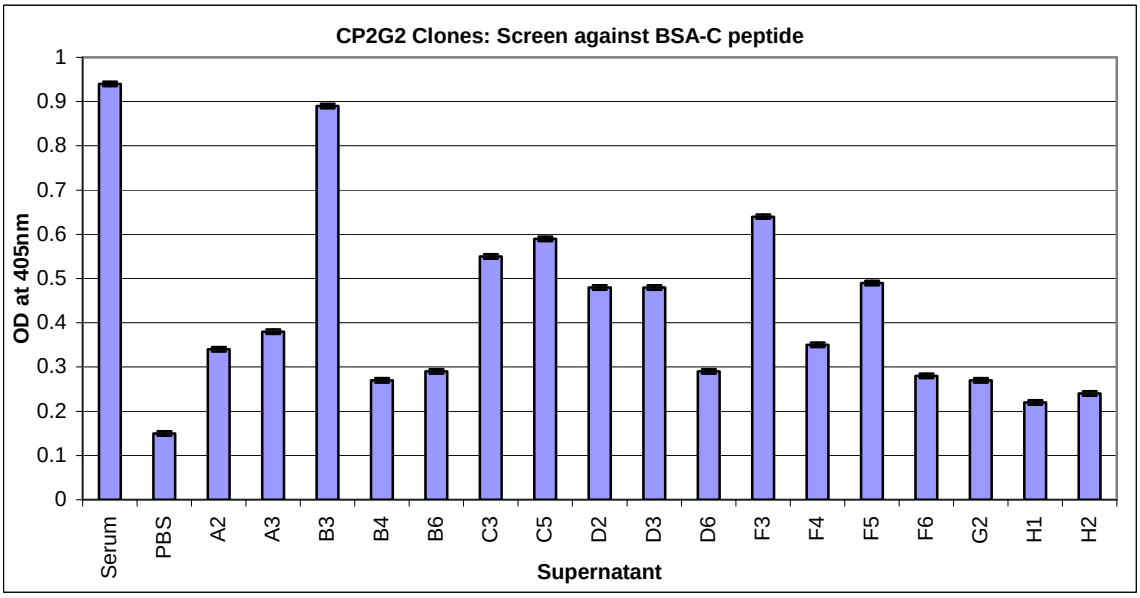
Hybridoma supernatants were then screened via flow cytometric analysis for recognition of native antigen expressed on 791T cells (**Figure 3.14**). 5 x 10^5 791T cells were coated with 791T/36, BRIC 216, PBS and 50µl hybridomas supernatant. 791T/36 and BRIC 216 both show a high level of CD55 recognition compared to unlabelled cells. All clones produce results comparable to background M.L.F values, indicating no recognition of conformational CD55 by the generated antibodies. Clone CP2 G2 showed weak binding, potentially indicating limited recognition and was cloned across a 96 well plate in order to produce a monoclonal antibody. This population was allowed to grow for several days and finally screened against C-BSA peptide to assess for antibody producing clones. Only wells containing viable cells were analysed (**Figure 3.15**).

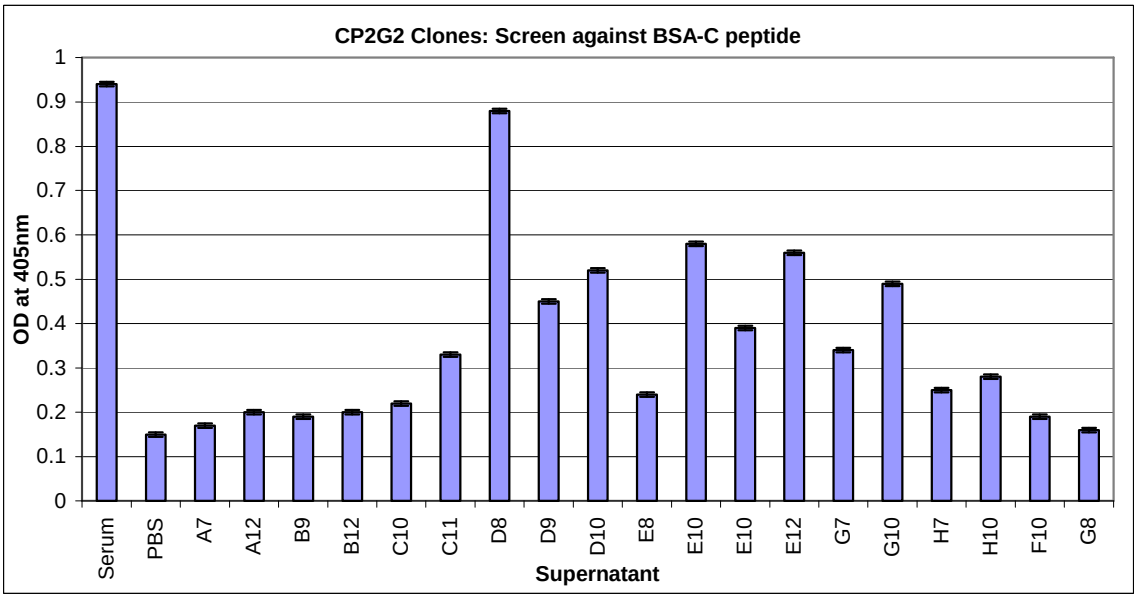
Figure 3.14: C fusion hybridoma positives screened against whole native CD55 via FACS analysis.
5 x 10^5 791T(CD55 expressing) and colo 205(non CD55 expressing) cells coated with 50µl hybridoma supernatant and incubated for 1 hour at 4ºC. Secondary rabbit anti mouse-FITC conjugate added and incubated for 1 hour at 4ºC. Cells analysed by flow cytometry and deviation shows two data points.



In **Figure 3.15**, sera produced strong OD values compared to PBS background control. All viable cells show degrees of peptide recognition, which is difficult to assess due to the number of cells present and speed of antibody production. CP2 G2: B3 and D8 produced the greatest responses to target peptide.

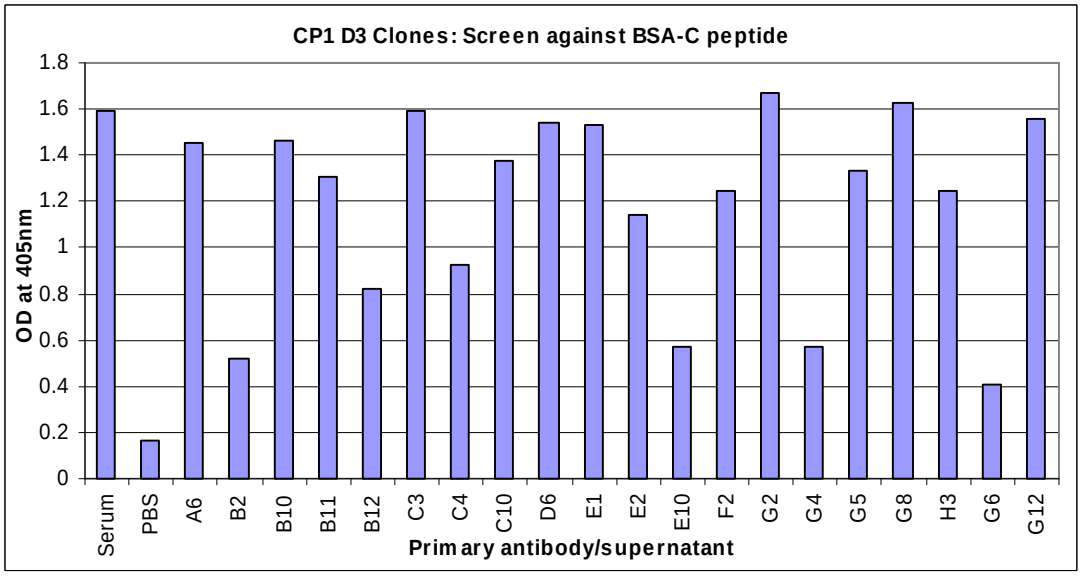
Figure 3.15: CP2 G2 Clones: Screen for presence of peptide specific antibody.
96 well plates coated with 5µg/ml BSA-C peptide overnight at 4ºC. 50µl of hybridoma supernatants added as primary antibody and secondary rabbit anti mouse HRP conjugate added and incubated for 1 hour at room temperature. Plates washed and ABTS substrate system used for ELISA detection. Plates read at 405nm and deviation expressed as two data points.





CP1 D3 clones were screened for peptide specificity (**Figure 3.16**). Sera and PBS controls produce expected results, with all hybridoma supernatants producing significantly elevated peptide recognition levels compared to negative controls (Increases of up to 5 fold responses).

Figure 3.16: CP1 D3 Clones: Screen for presence of peptide specific antibody.
96 well plates coated with 5µg/ml BSA-C peptide overnight at 4ºC. 50µl of hybridoma supernatants added as primary antibody secondary rabbit anti mouse HRP conjugate added and incubated for 1 hour at room temperature. Plates washed and ABTS substrate system used for ELISA detection. Plates read at 405nm and deviation expressed as two data points.



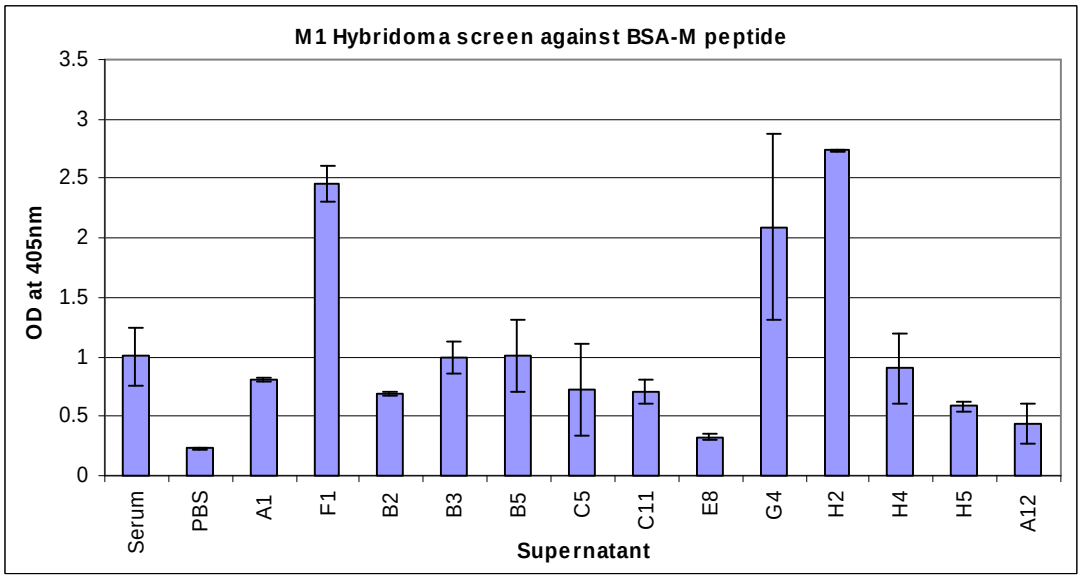
Supernatants from many clones were stored at 4°C and cell samples were stored at -163°C in liquid nitrogen. Cells were stored for potential later expansion and sub-cloning.

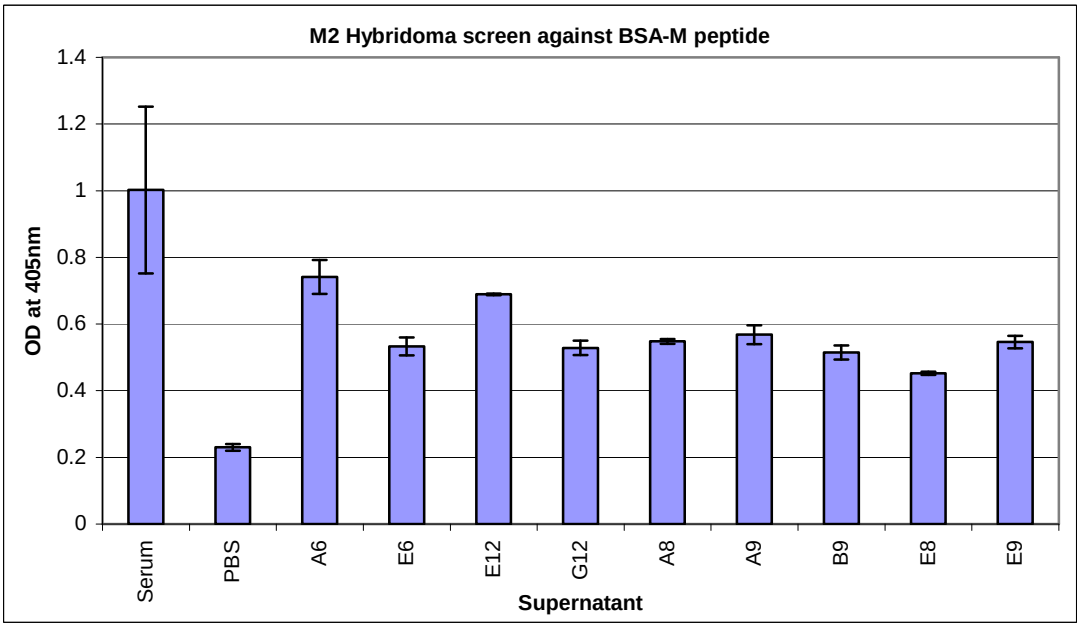
In conclusion, C fusion hybridoma screens indicate strong antibody specificity to linear peptide but not conformational antigen.

3.9: M Hybridoma screens and development

Mice M1 and M2 fusions were also screened for peptide specific antibody production, recognition of native antigen and potential CD55 blocking ability. **Figure 3.17** represents M1 and M2 hybridoma supernatant screen against BSA-M conjugated peptide, assayed by ELISA. Serum produced a strong positive OD value of 1.0, while only clones M1: F1, G4 and H2 gave greater responses to peptide. However, all other clones produced values ranging from 0.4 to 1.0, indicating the presence of antibody production. Results are difficult to assess due to potential rate of antibody production into supernatants, which could vary between different clones, also the presence of non-producing clones could reduce the comparative total antibody concentration.

Figure 3.17: Screen of M1 and M2 hybridomas for production of peptide specific antibody.
M1 and M2 splenocytes fused with NSO cells and selectively grown in HAT media. 96 well plates coated with 5µg/ml BSA-M peptide overnight at 4ºC. 50µl of hybridoma supernatants added as primary antibody and secondary rabbit anti mouse HRP conjugate added and incubated for 1 hour at room temperature. Plates washed and ABTS substrate used for ELISA detection. Plates read at 405nm and deviation expressed as two data points. Only data for wells containing viable cells in displayed.

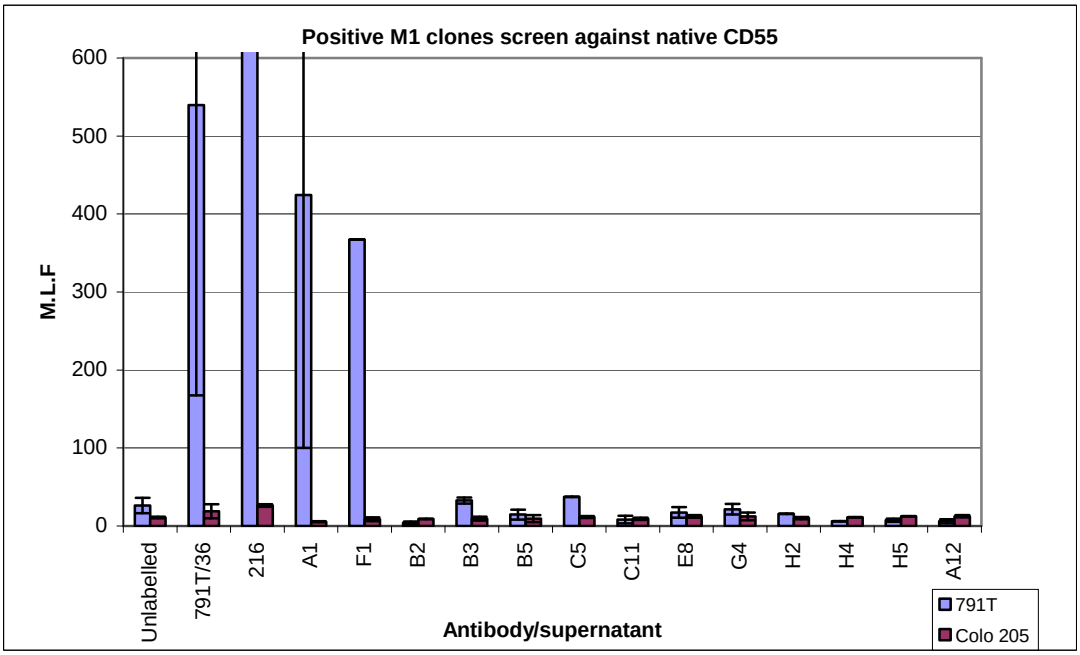




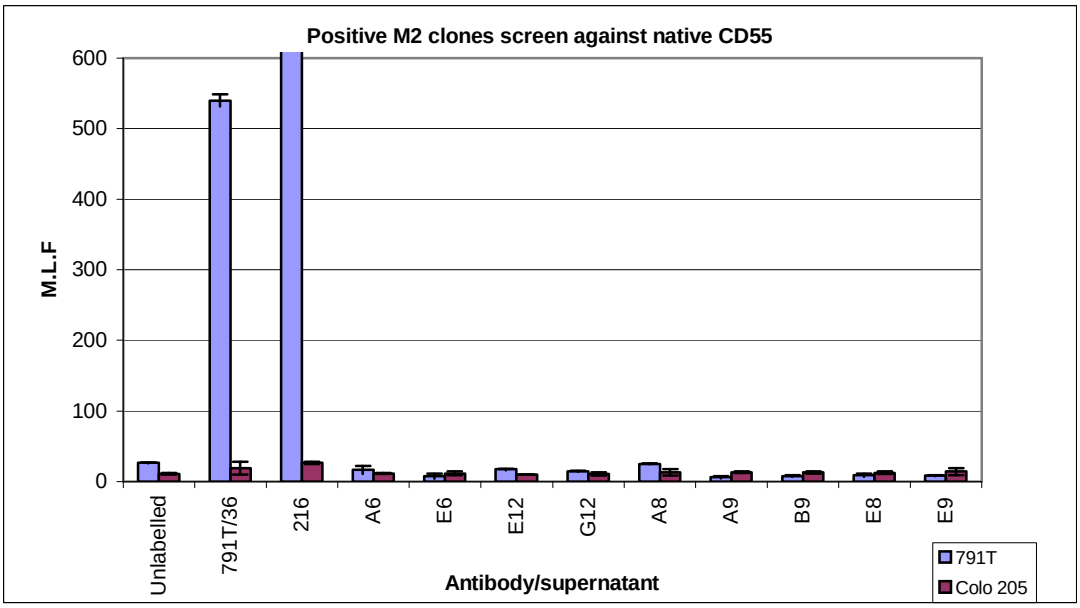
All viable clones assayed for peptide specificity were analysed for recognition of complete native antigen as expressed on 791T cells and results were obtained via flow

cytometry. **Figure 3.18** shows results for M1 and M2 clones, 791T/36 and BRIC 216 were used as positive controls and gave results 540 and 1083 (M.L.F) respectively. All other clones except M1: A1 and F1 gave results comparable with unlabelled control, indicating that most of the antibodies produced show no recognition of native antigen. While M1 A1 clone produced an M.L.F value of 400, the deviation produced was also significant, therefore only the M1 F1 clone was sub-cloned across a 96 well plate at 0.3 cells per well.

Figure 3.18: Screen of M1 and M2 peptide positive clones for recognition of native CD55 on 791T cell via flow cytometric analysis.
5 x 10^5 791T(CD55 expressing) and colo 205(non CD55 expressing) cells coated with 50µl hybridoma supernatant and incubated for 1 hour at 4ºC. Cells washed by centrifugation in PBS and secondary rabbit anti mouse-HRP conjugate added and incubated for 1 hour at 4ºC. Cells analysed by flow cytometry and deviation shows two data points.



216: 1083.28 +/- 425.69



216: 1083.28 +/- 425.69

Figure 3.19 represents analysis of M1 F1 clones screened against BSA-M peptide and indicates that most of the viable cells produced during the cloning process are generating antibodies. Antibody supernatants were then assessed by cytofluorimetric analysis for recognition of conformational CD55 as expressed by 791T cells (**Figure 3.20**).

Figure 3.19: Screen of M1 F1 sub clones against BSA-M peptide and purified CD55.

Supernatants screened via ELISA for presence of peptide specific antibodies. 5µg/ml BSA-conjugated M peptide and purified CD55 were coated on 96 well plates overnight at 4ºC. 50µl of hybridoma supernatant was added as primary antibody and secondary rabbit anti-mouse HRP conjugate was added and incubated for 1 hour prior to ABTS substrate addition. Plates were read at 405nm. Only data from wells containing viable cells shown.

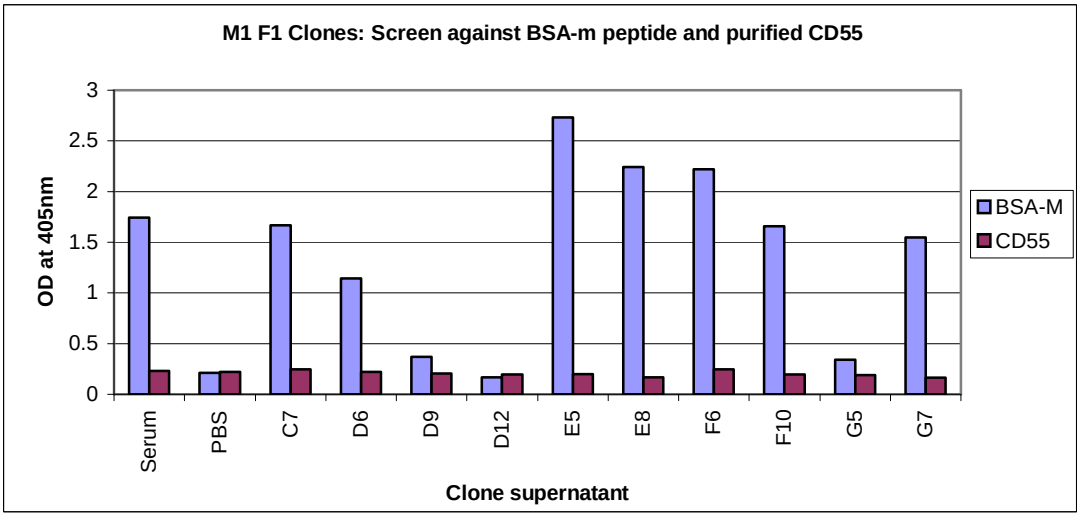
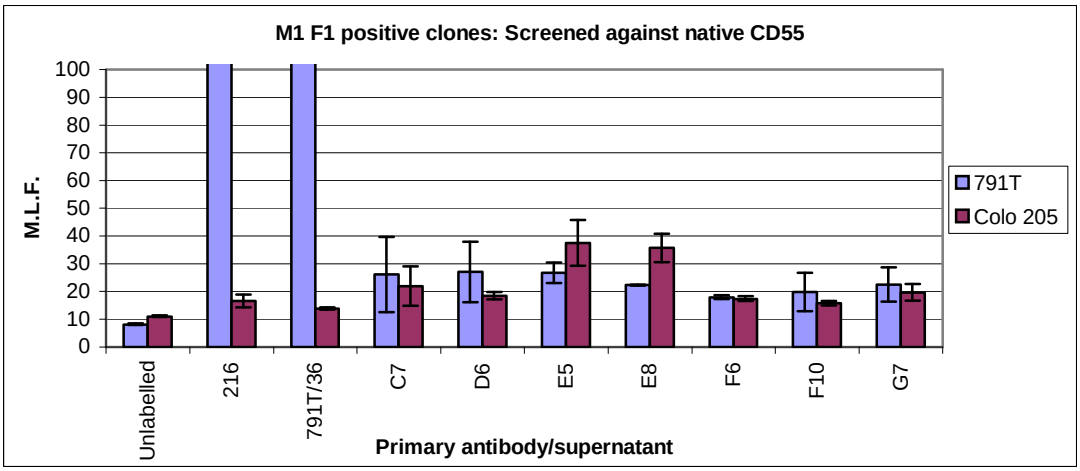


Figure 3.20: Cytofluorimetric analysis of M1 F1 peptide positive clones: recognition of conformational CD55 expressed on 791T cells.
5 x 10^5 791T(CD55 expressing) and Colo 205(non CD55 expressing) cells coated with 50µl hybridoma supernatant and incubated for 1 hour at 4ºC. Cells washed by centrifugation in PBS and secondary rabbit anti mouse-FITC conjugate added and incubated for 1 hour at 4ºC. Cells analysed by flow cytometry and deviation shows two data points.



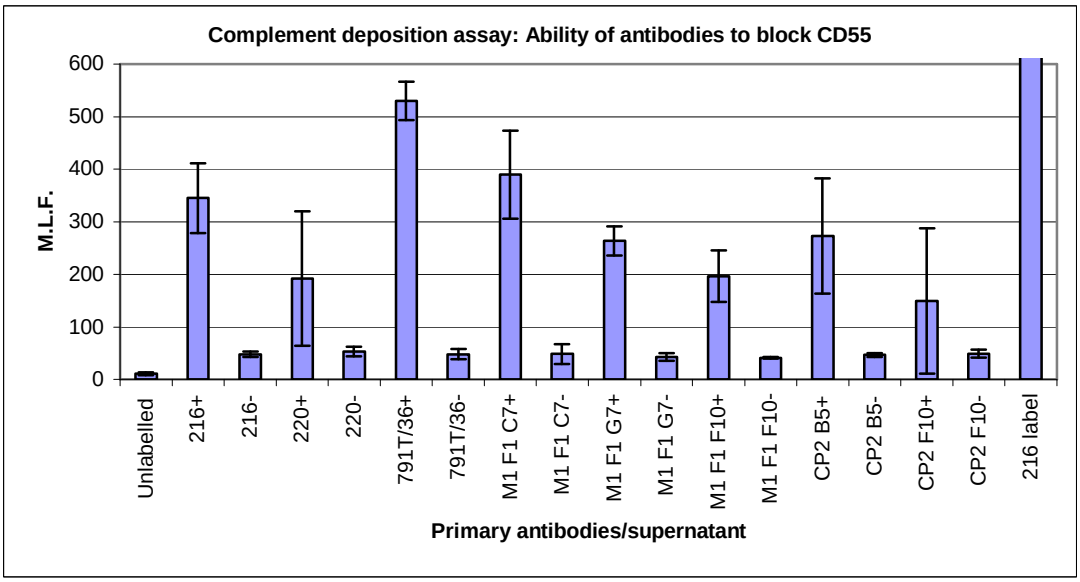
216: 2069.31 +/- 130.72 791T/36: 1776.25 +/- 1.92

BRIC 216 and 791T/36 used as positive controls gave M.L.F values greater than 1000, demonstrating the high expression level of CD55 upon the cell surface. All hybridomas produced results on the range of 25 to 35 for both 791T and Colo 205 cells, indicating no significant specificity for 791T cells over Colo 205 (non-expressing). These findings would imply that like the C-hybridomas, the M clones are also producing antibodies which only recognise linear peptide and possibly denatured antigen, but not native conformation. All supernatants were also assessed for their CD55 blocking ability. **Figure 3.21** represents complement deposition assay results using 791T cells, utilising supernatants from all strong M and C peptide positive hybridoma clones. 1 x 10^5 791T cells were labelled with BRIC 216 as positive control, blocking SCR3 domain of CD55 leading to increased C3b/c deposition. 791T/36(target SCR1-2) was also used as potential control along with BRIC 220 and unlabelled cells. All samples include cells without human serum as a negative control. All values produced were comparable with unlabelled negative control. BRIC 216 produced increased C3b deposition compared to control results, indicating that a degree of CD55 blockade is occurring. BRIC 220 showed a basal level of C3b deposition as expected, as it binds to SCR domain 1 and should not affect CD55 regulatory activity. 791T/36 showed unexpected results, as C3b deposition results were greater than those produced by BRIC 216, >500 and 300

respectively. 791T/36 targets domains 1-2 of CD55, and while SCR2 is involved in some classical and alternative pathway regulatory activity, a result greater than 216 blockade is not expected. Most hybridoma supernatants produce results comparable with BRIC 220 indicating no alteration in CD55 activity and thus no blocking effect. The M1 F1 C7 clone does produce an M.L.F value similar to BRIC 216 and may therefore have some inhibitory effect upon CD55 activity.

Figure 3.21: Strongest peptide positive hybridoma supernatants screen for CD55 blocking ability.

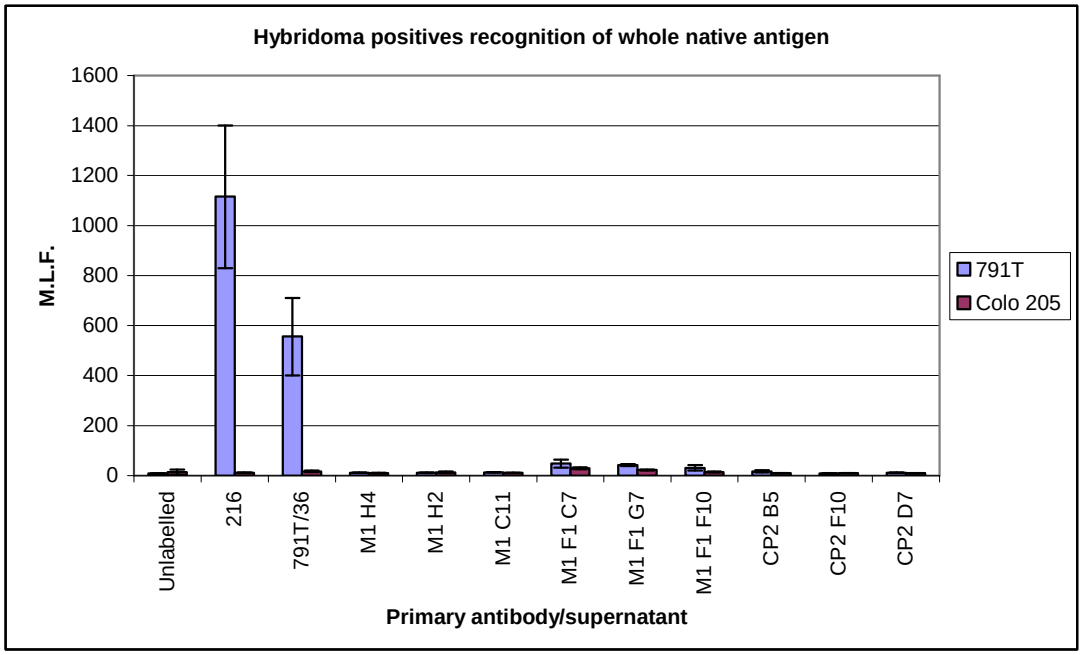
1 x 10^5 791T cells labelled with BRIC 216, 791T/36, BRIC 220 and 50µl hybridoma supernatant. Cells were incubated for 2 hours at 37°C with human donor serum obtained from three individuals. Polyclonal anti human C3c FITC conjugated antibodies were added and incubated for 1 hour at 4°C. Cells were assayed by flow cytometry and deviation shows two data points.



216 labelled alone: 3240.4 +/- 71.98

Figure 3.22 represents a screen of supernatants obtained from hybridomas previously assayed for decay accelerating factor blocking ability. This assay was carried out to confirm if produced antibodies bound to native CD55, screened against 791T cells by indirect FACS analysis. All clones produced limited responses with values comparable with unlabelled cells, indicating no recognition of native antigen.

Figure 3.22: Screen of strongest hybridoma peptide positives for whole native antigen recognition.
5 x 10^5 791T(CD55 expressing) and Colo 205(non CD55 expressing) cells coated with 50µl hybridoma supernatant and incubated for 1 hour at 4ºC. Cells washed by centrifugation in PBS and secondary rabbit anti mouse-HRP conjugate added and incubated for 1 hour at 4ºC. Cells analysed by flow cytometry and deviation shows two data points.



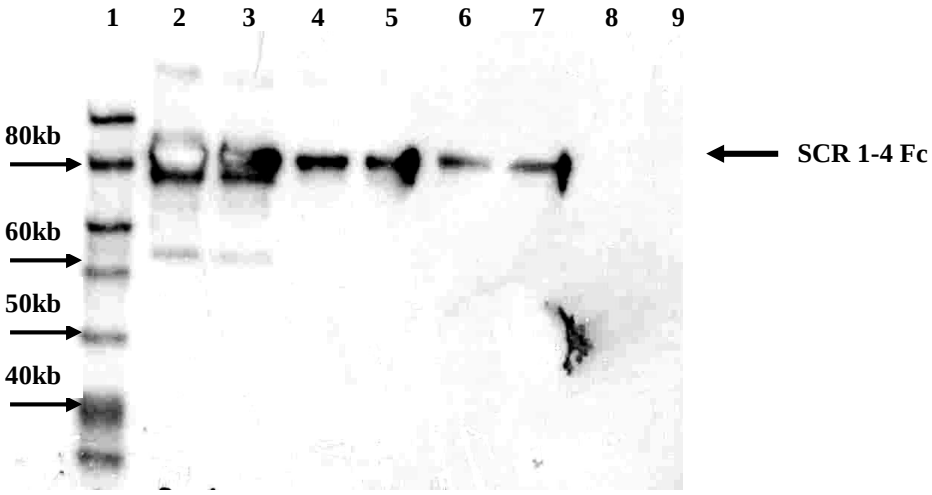
Results obtained indicate that immunisation with target peptides produce strong responses from B cells specific for the linear peptide sequence. However, it appears that the natively expressed CD55 molecule on target tumour cells does not expose the same sites as the peptide. This may be due to conformational tertiary structure masking linear peptide binding sites. The predicted three dimensional models of peptide sequences as shown in **Figure 3.3**, display predicted native conformation based on the SCR domains of CD46. These models incorporate the post translational modifications of naturally expressed proteins which are not generated by the target peptides used.

3.10: Assessment of RM1 monoclonal antibody

Peptide positive clones have been repeatedly cloned, and monoclonal populations expanded to generate high antibody volumes and concentrations. Clone M1 F1 F10 was expanded, and a stable cell line was generated expressing high levels of antibody specific to the M peptide, having been derived from the human CD55 sequence. This population was chosen for large scale production of anti-CD55 antibodies as it

exhibited consistent specificity, as assessed by ELISA, and stability. This antibody was designated RM1. **RM1** antibody was purified via protein G affinity column and glycine release protocols. Purified antibody was tested via peptide ELISA, western blot analysis and direct 791T labelling and assessment by FACS analysis to determine specificity and functionality. Recombinant SCR1-4 Fc fusion protein (Harris C 2001), used to represent structurally native CD55, was electrophoresed on an 8% SDS-ploy acrylamide gel under non-reducing conditions and transferred to a nitrocellulose matrix. The blot was probed with anti-CD55 antibodies including RM1 (**Figure 3.23**). BRIC 216, 791T/36 and RM1 all recognised a band approximately 80kDa in size, equating to SCR 1-4 Fc. The control lane, without addition of primary antibody confirms presence of specific binding. Protein analysed by western blotting in non-reducing conditions will be partially denatured by the presence of the ionic detergent SDS, which may alter SCR 3 conformation sufficiently for RM1 recognition.

Figure 3.23: Western blot analysis of SCR 1-4Fc fusion protein.
50μg/ml recombinant CD55 [SCR1-4Fc (Harris C. 2000)] electrophoresed on 8% SDS-polyacrylamide gel under non-reducing conditions and transferred to nitrocellulose matrix. Blot probed with 2μg/ml anti-CD55 antibodies and developed with ECL detection reagents.



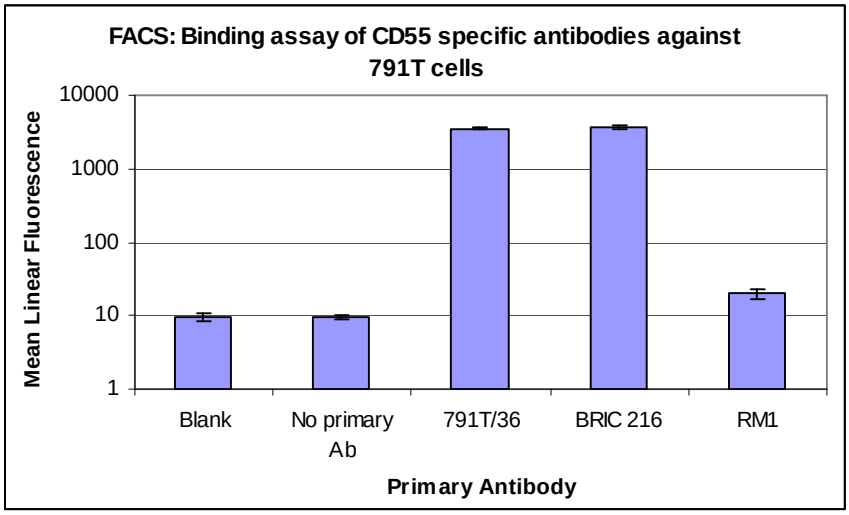
Lanes:	1	Molecular weight markers
	2 + 3	BRIC 216 (SCR3)
	4 + 5	791T/36 (SCR1-2)
	6 + 7	**RM1 (SCR3)**
	8	Control lane (No primary antibody)
	9	Control lane 708 (Non specific mouse antibody)

Cytofluorimetric analysis of RM1 binding to CD55 as expressed on 791T cells (**Figure 3.24**) indicates that there is limited recognition of conformational antigen.

RM1 antibody produces mean linear fluorescence comparable to control samples while BRIC 216 and 791T/36 both produce fluorescence values up to 2 log values greater.

Figure 3.24: FACS analysis of purified RM1 specificity to native conformational CD55 as expressed on 791T cells.

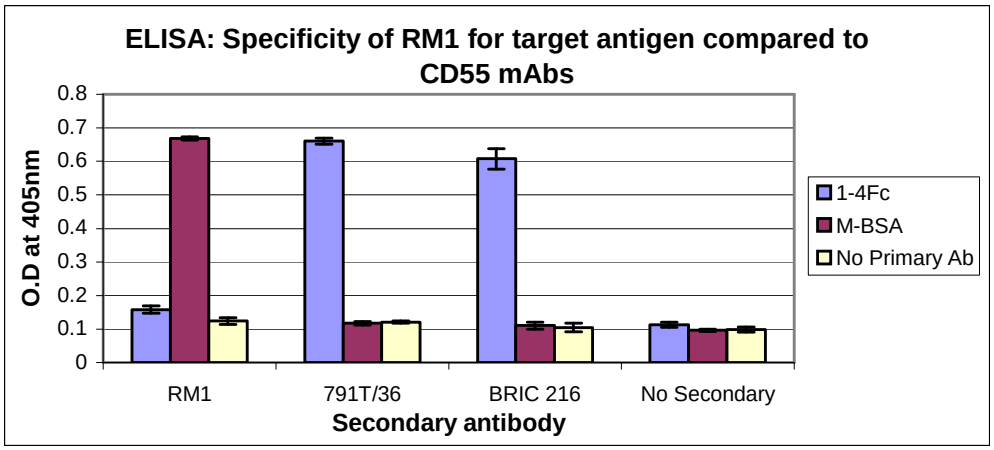
1 x 10^5 791T cells labelled with 1µg primary antibody (RM1, 791T/36 and BRIC 216) for 1 hour at 4°C. Cells were incubated with secondary rabbit anti mouse FITC conjugated antibody for 30 minutes and cell samples were assessed by flow cytometry. Standard deviation is expresses as two data points.



To finally confirm RM1 specificity, a 'sandwich' ELISA was used to assess recognition of native protein (SCR 1-4Fc) and target peptide (M-BSA). It can be inferred from **Figure 3.25**, that while RM1 has high specificity for M-BSA peptide (O.D of 0.65), limited recognition of recombinant protein is achieved (O.D of 0.15), which is comparable to negative control values. It is worth noting that neither BRIC 216 or 791T/36 recognise M-BSA peptide while both show high OD values for the complete structural protein, suggesting that BRIC 216's target epitope is part of the tertiary structure of CD55 and not linear peptide.

Figure 3.25: Assessment of RM1 antigen specificity via ELISA.

5µg/ml target antigen was coated on a 96 well plate and specific binding of several anti-CD55 antibodies was assessed. ABTS substrate was used and results were obtained via a plate reader. All samples were run in triplicate producing standard deviation expressed as two data points.

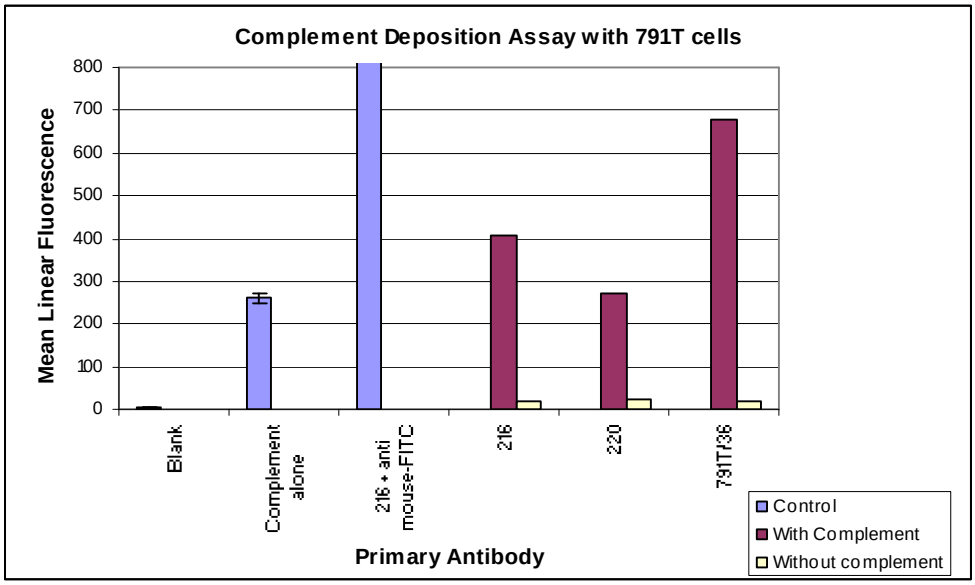


3.11: Assessment of 791T/36 inhibition of CD55 decay accelerating activity

As 791T/36 showed novel results in the complement deposition assay, the procedure was repeated to produce consistent results. **Figure 3.26** represents the results obtained from C3b/c deposition on 791T cells when CD55 inhibition is attempted with BRIC 216, BRIC 220 and 791T/36. BRIC 216 was also used to demonstrate the presence of CD55 on the cell surface, producing M.L.F of 3287. BRIC 220 shows binding of SCR1 of CD55 and no inhibition of regulatory activity, M.L.F of 260, while BRIC 216 binding to the SCR3 domain produces increased deposition giving values of up to 400. 791T/36 however, produces values of 650, displaying a significant increase in the level of complement deposition and therefore CD55 inhibition. 791T/36 affects CD55 regulatory activity without binding directly to SCR3, which has been shown in deletion mutants to generate the greatest defects in CD55 function compared to other domains. It is proposed that 791T/36 inhibits CD55 function either by steric hindrance, masking the main active site, or by inducing alteration of conformational structure upon binding to SCR1-2.

Figure 3.26: Repeat of C3b/c deposition assay for 791T/36 blocking ability compared to BRIC 216 and BRIC 220.

1 x 10^5 791T cells labelled with BRIC 216, 791T/36, BRIC 220. Cells were incubated for 2 hours at 37°C with human donor serum obtained from three individuals. Polyclonal anti human C3c FITC conjugated antibodies were added and incubated for 1 hour at 4°C. Cells were assayed by flow cytometry and deviation shows two data points.



BRIC 216 + anti-mouse FITC: 3287.95

3.12: Discussion

The initial aim of this project was to produce an antibody specifically targeting the active site of CD55 and inhibiting its decay accelerating activity. As mentioned, previous studies have identified specific domains within CD55 that maintain its function. Deletion mutants have shown that SCR domain 3 plays a pivotal role in disrupting both the alternative and classical complement pathways. This factor identified SCR 3 as a valid target for monoclonal antibody development. Preliminary peptide immunisation strategies were chosen in order to direct a response towards the specific epitope within the SCR domains of CD55.

Through utilisation of hydrophobicity algorithms, the hydrophilic pattern of SCR 3 protein structure was determined. Comparison of the hydrophilic domains within the sequence against the crystal structure of CD46 enabled prediction of the surface locations of these specific peptides. These regions from within the SCR 3 peptide sequence, identified as being exposed on the protein surface, are potentially antigenic and were therefore successfully synthesised and coupled to carriers to be used in the immunisation protocol. A preliminary strategy for immunisation was to prime and boost responses with the peptides in order to generate a B cell population specific for the target sequences. Later boosting of responses could be achieved through immunisation with whole cells, where the only common antigens would be regions within the SCR 3 domain of CD55.

These results would implicate that a preliminary peptide immunisation strategy only directs antibody development specific for the linear peptide. Peptide synthesis does not incorporate post translation modifications that occur in vivo, and therefore generation of native tertiary structure is not produced. Thus native epitopes are not generated and responses produced are towards the linear structure. Craig et al (1998) state that when polyclonal antibodies are screened against peptide libraries, the positive peptide sequences isolated recognise linear regions within the complete protein structure. This would indicate that peptides are able to generate protein specific antibodies as long as the specific epitopes tertiary structure is a linear region within the protein. Wilson et. al., (1995) state that antibodies specific to peptides interact with their target antigen differently to protein specific antibodies and involve

the presence of ionic interactions, Van der Waal's forces and H bonds. While antibodies possess only limited canonical conformations, their binding sites are specific to their antigens. Protein specific antibodies possess large, flat binding sites that enable primary 'docking', which then induces conformational changes within both the antibody and the antigen, thus exposing previously hydrophobic residues, generating high affinity interactions by increasing complementarity and charge based interactions (Braden et al, 1995 & Lara-Ochoa et al, 1996). However, peptide specific antibodies possess smaller, deeper clefts in which peptide sequences bind, being unable to easily bind protein structures and only with weak affinity. It appears that to generate successful cross recognition from peptides to proteins, small sequences from within linear domains of proteins are required. The initial peptide predictions used in this study produce 'barrel' like conformations and hence the initial docking affinity for complete structural epitopes may be weak. Therefore, to improve peptide strategies, short sequences containing residues with strong binding potential should be used. This could be achieved by following methodology used by Craig et. al. (1998), utilising more superpositional structural assignment computer programs. This would aid in the evaluation of conformational preference inherent in each sequence, enabling identification of structural domains in the peptide that match protein epitopes.

These findings show a functional use for RM1 as an antibody detecting the presence of CD55 under varying conditions. One particular area of importance is for staining of paraffin embedded tissue sections, which has been assessed by Dr Zahra Madjd (2004). There are currently no good anti-CD55 antibodies available for immunohistochemical analysis. Madjd et. al., have shown utilising RM1, that in a study of 480 patients with primary breast carcinoma, high expression of CD55 is associated with low histological grade and good prognosis and that loss of CD55 is associated with poor prognosis. CD55 expression in breast carcinoma is also comparable with CD59 expression, and while these findings are controversial, they suggest further roles for CD55, this providing more evidence of the potential benefit from targeting CD55 in tumour therapy. 791T/36 mAb has been used to assess CD55 expression on colorectal cells via FACS analysis, with increased antigen expression associated with poor prognosis (Durrant et al, 2003). These findings would be predicted, although it is unclear whether CD55 acts predominantly to suppress

complement mediated attack or as an inhibitor of T cell specific responses via CD97 (Durrant et al, 2003). It appears that CD55 acts via numerous pathways and its expression is involved with many interactions.

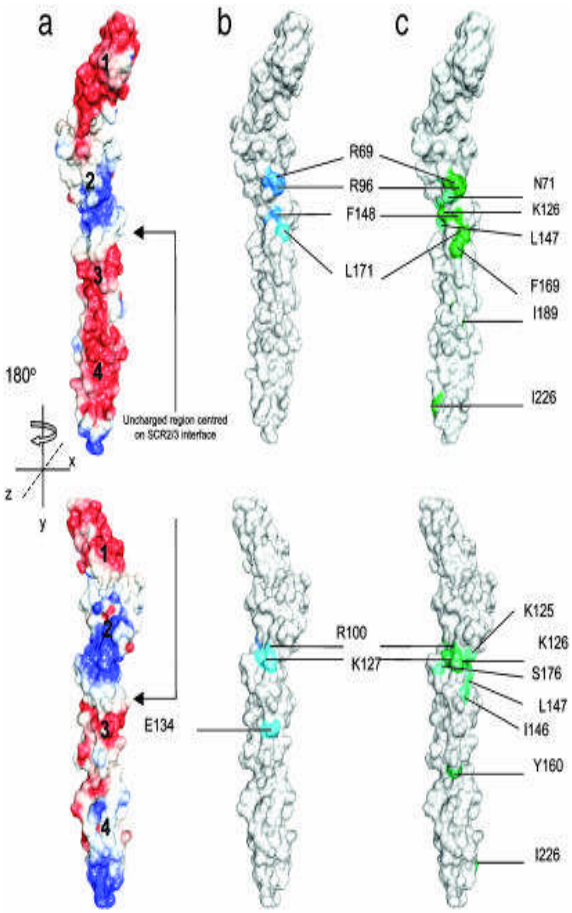
Finally, during complement deposition assays, it was noted that 791T/36 which specifically binds SCR domains1-2 of human CD55 significantly affected the level of C3b deposition produced. These findings indicate that while 791T/36 does not directly bind the active SCR 3 site of CD55, it potentially either blocks the SCR3 via steric hindrance or it induces a conformational change within the CD55 structure itself, preventing decay accelerating activity. Results obtained indicate that 791T/36 binds native CD55 on the surface of osteosarcoma cells with similar ability as BRIC 216, a SCR3 targeting antibody. When compared via ELISA against recombinant protein, 791T/36 generates a greater response than BRIC 216, and also compared via complement deposition assay against 791T osteosarcoma, the anti SCR 1-2 appears to enhance C3b deposition with greater ability compared to BRIC 216. In order to determine whether increased complement deposition was indeed mediated by the inhibition of CD55 decay accelerating activity, or whether by antibody fixation of complement, further analysis must be carried out. This could be achieved with the use of C1q depleted serum, which would remove the complement component, which binds to the Fc domains of antibodies used within this analysis. By incorporating this step, any enhanced $C3_{b/c}$ deposition observed would be due to blockade of CD55's active site.

The decay accelerating activity of CD55 has been located within domains 2-3 for classical pathway and 2-4 for alternative pathway activity (Brodbeck et al, 1996). Mutations within SCR 3 however, have produced the greatest disruption of CD55's regulatory activity, although SCR 1-2 transfectants have generated regulation of the alternative complement pathway (Christiansen et al, 2000).

A recent paper by Lukacik et al (2004) analysing the crystal structure of CD55 has elucidated several inconsistencies in prior literature. They state that the previous studies show that interaction between CD55 and the convertases were likely to involve large areas on the surface of CD55 (classical pathway convertases contact SCR 2 and 3, whereas alternative pathway convertases contact SCR 2, 3 and 4).

However, mapping of the key interaction site for both convertases has been complicated by the fact that the site is centred on residues located at the junction between SCR 2 and 3. Lukacik et al state that many of these residues only act indirectly by structural perturbation of CD55 at the SCR2/3 interface.

Mapping of biological function onto CD55 structure:
Lukacik et al (2004)



Two views of CD55 are shown folded by 180° rotation about the vertical axis.
(a) Charge distribution is coloured from blue (positive) to red (negative), via white (no charge).
(b) Mapping of amino acid positions where substitution is known to affect classical pathway regulatory activity. Positions where substitutions reduces activity to 10% or less of wild type activity (Dark blue), and 50% or less (Light blue).
(c) Mapping of amino acid positions where substitution affects alternative pathway convertases to 10% or less of wild type activity (dark green), and 50% or less (light green).

They also state that functional protein-protein interactions depend on a combination of affinity and high specificity, often involving polar (hydrogen bonding and electrostatic) and apolar (Van der Waals and hydrophobic) contacts. Their analysis of hydrophobic surface potential suggest a strong candidate for a protein binding site centred on Leu 171 and Phe 169 near the SCR 1/2 interface. These have been identified previously, showing that substitutions in Leu 171 decrease CD55 regulatory ability of both convertases while mutations at Phe169 decrease AP convertase regulation (Kuttner-Kondo, 2001).

This data would indicate that an antibody raised to a linear peptide of SCR3, while useful as a CD55 probe, is unlikely to possess CD55 neutralising capability. However, as 791T/36 has shown promising results, this new molecular target data may strengthen the hypothesis that this SCR1-2 specific antibody may at least be involved in perturbation of CD55 structure or may act by steric hindrance, blocking the SCR1/2 domain interface.

Spendlove et. al., (2006) have demonstrated that 791T/36 is capable of blocking the interaction between CD55 and CD97 on activated T cells, thus preventing CD55's inhibition of T cell function. These findings suggest that 791T/36 as a SCR 1-2 directed antibody has the potential to not only inhibit CD55 as a regulator of complement but also as a blocker of T cell responses. This would suggest that 791T/36 could be used to potentially prevent CD55 activity and sensitise target tumour cells to autologous complement and enhance a cell specific T lymphocyte response. This can be achieved by humanising 791T/36, as previously discussed.

For many years 791T/36 was used for imaging of a variety of tumours and showed effective localisation in patients with bone and soft tissue sarcomas (Armitage et al, 1986). As CD55 is also present on most normal cells this could be a potential problem. However, on osteosarcoma, ovarian, gastric and colorectal cells, CD55 is present at 10^5 to 10^6 molecules per cell (Spendlove et al, 1999) compared to 10^4 molecules on red blood cells, which has been shown to be sufficient for complement protection. Within the tumour environment, its elevated expression allows divalent, higher affinity binding compared to low antigen levels allowing weak monovalent interactions upon erythrocytes. It is this high-density expression within tumours that is favoured and enables antibody accumulation on the tumour surface. This has been shown with patients treated with radio labelled 791T/36 showing only background levels of radioactivity on peripheral blood mononuclear cells and red blood cells. Pimm et. al., (1987) stated that 791T/36 not only was useful for immunodetection of primary and colorectal carcinomas but also showed potential for targeting of therapeutic agents. 791T/36 was coupled to methotrexate, vindesine and daunomycin, and these conjugates retained drug activity, antibody function and localised to colorectal carcinoma xenografts.

All of these factors indicate a potent use for 791T/36 and other SCR 1-2 targeting antibodies. Humanisation of this mouse IgG2b could enable it to be used therapeutically to block CD55 function and target it as a tumour associated antigen. This would function by recruiting complement and T cells responses as well as potentially sensitising cells to $CD8^+$ and NK cell attack. Other therapeutic antibodies used for the treatment of tumours, such as rituximab, have also shown to be more effective when used in combination with complement regulatory protein blocking antibodies (Golay et al, 2000). The biotechnology company Viragen have humanised 791T/36 and are currently assessing its potential as a stand alone anti-cancer therapy. Preclinical trials are also underway determining the use of this CD55 inhibitor in combination with other anti cancer antibodies in order to promote anti tumour responses. In March 2005, Viragen published a press release stating that their CD55 specific antibody was found to significantly enhance cancer cell destruction mediated by the drug Rituxan® by *in vitro* assessment.

Chapter 4: Development and assessment of SCR 1-3 Fc DNA vaccine eliciting antibody responses to CD55

4.1: Introduction

As our previous peptide immunisations generated antibodies recognising only linear peptides, we decided to immunise with functional protein to generate CD55 neutralising antibodies. As our own data conforms with current research indicating than CD55 activity localises to the SCR 1-2 interface and that previous studies have shown that complement regulation may involve SCR domain 3, new CD55 immunisation strategies were developed. DNA constructs were generated incorporating SCR 1-3 domains of CD55 into the Signal pIg plus vector, aiming to generate SCR1-3 Fc fusion proteins. This strategy was used in order to develop native, functional protein, which could be used both as vaccine and as potential screen for antibody responses. The DNA constructs could also be used to immunise directly via Helios© gene gun, with the aim of directing *In vivo* expression of functional protein either by cellular uptake or through direct transfection of antigen presenting dendritic cells or by DC uptake from transfected keratinocytes.

Post DNA immunisation, antigens are presented to the immune system by two main mechanisms for the development of antigen specific CTL responses, namely direct priming and cross presentation. Direct priming requires DNA transfection of APCs, whilst cross presentation involves antigen expression within somatic cells, which are in turn presented to APCs. Cross presentation, mediated by bone marrow derived APCs, enables both processing and presentation of exogenous antigens in an MHC class I restricted manner. It is this process which has been identified as essential in the generation of CTLs specific to tumour, virus and tissue antigens (Shen et. al., 2004). Development of cross priming can be mediated by both non-specific interactions, through pinocytosis, or can be mediated through specific receptors in an active process. Targeting of proteins to such specific receptors on APCs may enhance presentation and incorporation into the adaptive immune response. FcγRI is involved in active antigen uptake and transfer to endocytic compartments involved in

processing within APCs. Targeting antigen delivery to APC via FcγRI was achieved by combining target DNA to a human IgG1 tail.

DNA immunisation by gene gun utilises 100 times less DNA then alternative intramuscular vaccination, and is capable of producing equivalent immune responses. The increased reliability and reproducibility of gene gun modality indicates that this mode of administration could easily be translated into human trials in the form of potential anti-tumour therapy. As ballistic immunisation strategies have already been developed for vaccination into human epidermis, this strategy for generation of immunity is viable for translation from mouse models into a human system.

Subsequent DNA immunisations were carried out using gene gun protocols, and antibodies generated were assayed via ELISA specifically recognising the SCR 1-3 Fc fusion protein.

Antibodies generated by the SCR 1-3 Fc (Wild Type) construct are compared with those raised by immunisation with the same construct incorporating a heteroclitic epitope. **Chapter 5** details the rationale and methodology utilised for identification and development of the construct containing the CTL epitope Z158m. The results are combined in order to directly compare antibody responses mediated towards the wild type and mutated vaccines, and to determine what effects alteration of the native sequence has on immune responses generated.

Results

4.2: Development of SCR 1-3 Fc DNA vaccine

Human full length CD55 cDNA had been previously isolated from 791T osteosarcoma cells and TA cloned into the PCR II- TOPO vector. This plasmid is used as it incorporates a topoisomerase I enzyme allowing the direct insertion of *taq* polymerase amplified PCR products. Ligation products were then used to transform TOP10F competent bacteria and plated on to ampicillin selective LB agar plates. Single colonies were assessed for incorporation of PCR II TOPO plasmid using M13 forward and reverse primers and mini cultures of positive clones were grown in selective LB media. Qiagen DNA preparations were made and glycerol stocks of transformed cells were also made and stored at -80°C.

4.3: PCR amplification of SCR 1-3 products for ligation into Signal pIgplus vector.

Primers were designed to amplify the CD55 leader SCR 1-3 regions from PCR II-TOPO, incorporating restriction sites for *kpnI* in the forward primer and *BamHI* in the reverse primer. **Figure 4.1** displays the CD55 leader primer and SCR 3 reverse primer utilised for extraction of the desired sequence from complete CD55. The DSCR23 primer sequence is also displayed which was initially used for amplification of the SCR 2 domain of CD55 and was used in this case for sequence analysis. The *kpnI* restriction site had been incorporated into the Signal pIgplus vector via site directed mutagenesis along with removal of the factor Xa cleavage site. **Figure 4.2** shows full length CD55 sequence indicating primer binding sites for desired PCR product amplification. Determination of optimal annealing temperature for the primer set was determined using standard PCR protocols, varying the annealing temperatures used (**Figure 4.3**). Successful amplification was observed via analysis of reaction samples via visualisation on a 1% agarose gel; desired product is seen as a 697bp band. CD55 leader SCR1-3 PCR product was then ligated into an empty PCR II-TOPO vector, enzyme digested with *kpnI* and *BamHI*. DH5α competent cells were then transformed with the new ligation and cultured on ampicillin selective LB agar plates. Single colonies were again cultured and Qiagen maxi preparations were carried out to isolate the DNA construct (CD55 leader SCR1-3 PCR II-TOPO).

Figure 4.1: Primer Design for CD55 leader SCR 1-3 incorporation into Signal pIgplus plasmid

CD55 Leader Forward Primer 5'TT GTT [GGT ACC] CGG CGC GCC ATG ACC3' ***kpnI*** **Met Thr** Binds from 46-71bp of CD55 cDNA sequence	26mer TM = 66
SCR 3 Reverse Primer 5' G[GGATC]CGC TTC TCT GCA CTC TGG CAA CG 3' ***BamHI*** **Glu Arg Cys Glu Pro Leu** Binds from 740-712bp of the CD55 cDNA sequence	29mer TM = 72.6
DSR 23 Reverse Primer 5' [GG ATC C]TT ACA AAA TTC GAC TGC TGT GGA C3' ***Bam HI*** **Lys Cys Phe Glu Val Ala Thr Ser** Binds from 547-518bp of the CD55 cDNA sequence	29MER TM = 72.6

Forward and reverse primers designed to bind to CD55 leader sequence in the 5' direction and SCR 3 in the 3' direction. Amplification of desired sequence from combined primer set yields a fragment of 697bp.

Figure 4.2: Human CD55 DNA and Protein Sequence

DNA Accession No: BC001288
60-184 CD55 Leader
185-361 SCR 1
371-553 SCR 2
566-739 SCR 3
752-928 SCR4

Protein Acc No:P08174
35-95 SCR1
97-159 SCR2
162-221 SCR3
224-284 SCR4
287-356 SER/THR Rich
M Start of protein sequence

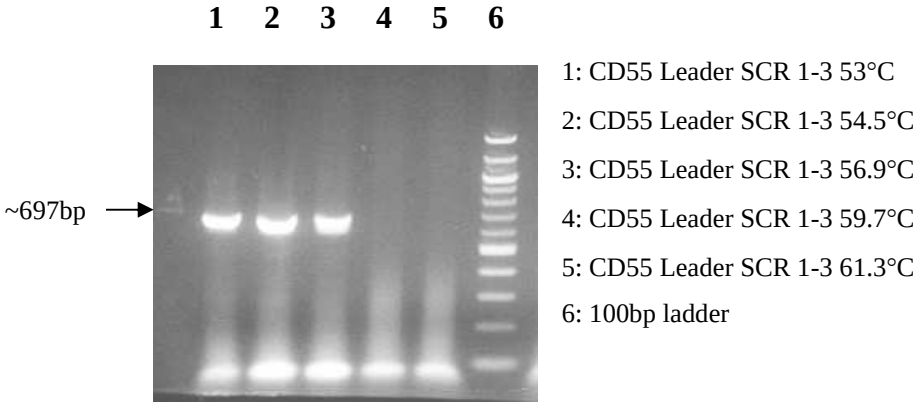
```
1   gcaactcgct ccggccgctg ggcgtagctg cgactcggcg gagtcccggc ggcgcgtcct
      Q  L  A   P  A  A  G   R  S  C   D  S  A   E  S  R   R  R  V  L
61  tgttctaacc cggcgcgcca tgaccgtcgc gcggccgagc gtgcccgcgg cgctgccccc
      V  L  T   R  R  A  M   T  V  A   R  P  S   V  P  A  A   L  P  L
121 cctcggggag ctgccccggc tgctgctgct ggtgctgttg tgcctgccgg ccgtgtgggg
      L  G  E   L  P  R  L   L  L  L   V  L  L   C  L  P  A   V  W  G
181 tgactgtggc cttcccccag atgtacctaa tgcccagcca gctttggaag gccgtacaag
      D  C  G   L  P  P  D   V  P  N   A  Q  P   A  L  E  G   R  T  S
241 ttttcccgag gatactgtaa taacgtacaa atgtgaagaa agctttgtga aaattcctgg
      F  P  E   D  T  V  I   T  Y  K   C  E  E   S  F  V  K   I  P  G
301 cgagaaggac tcagtgatct gccttaaggg cagtcaatgg tcagatattg aagagttctg
      E  K  D   S  V  I  C   L  K  G   S  Q  W   S  D  I  E   E  F  C
361 caatcgtagc tgcgaggtgc caacaaggct aaattctgca tccctcaaac agccttatat
      N  R  S   C  E  V  P   T  R  L   N  S  A   S  L  K  Q   P  Y  I
421 cactcagaat tattttccag tcggtactgt tgtggaatat gagtgccgtc caggttacag
      T  Q  N   Y  F  P  V   G  T  V   V  E  Y   E  C  R  P   G  Y  R
481 aagagaacct tctctatcac caaaactaac ttgccttcag aatttaaaat ggtccacagc
      R  E  P   S  L  S  P   K  L  T   C  L  Q   N  L  K  W   S  T  A
541 agtcgaattt tgtaaaaaga aatcatgccc taatccggga gaaatacgaa atggtcagat
      V  E  F   C  K  K  K   S  C  P   N  P  G   E  I  R  N   G  Q  I
```

601tgatgtacca ggtggcatat tatttggtgc aaccatctcc ttctcatgta acacagggta
D V P G G I L F G A T I S F S C N T G Y
661caaattattt ggctcgactt ctagtttttg tcttatttca ggcagctctg tccagtggag
K L F G S T S S F C L I S G S S V Q W S
721tgacccgttg ccagagtgca gagaaattta ttgtccagca ccaccacaaa ttgacaatgg
D P L P E C R E I Y C P A P P Q I D N G

CTAACC . CD55 leader primer binding site. Region in primer mutated to produce GGTACC = *kpnI* restriction site in Signal pIgplus vector

CCAG. SCR 3 reverse primer binding site incorporating *BamHI* site post Glutamic acid (E) position.

Figure 4.3: Optimisation PCR of CD55 Leader SCR 1-3 from PCR II TOPO construct. Analysis of primer set.



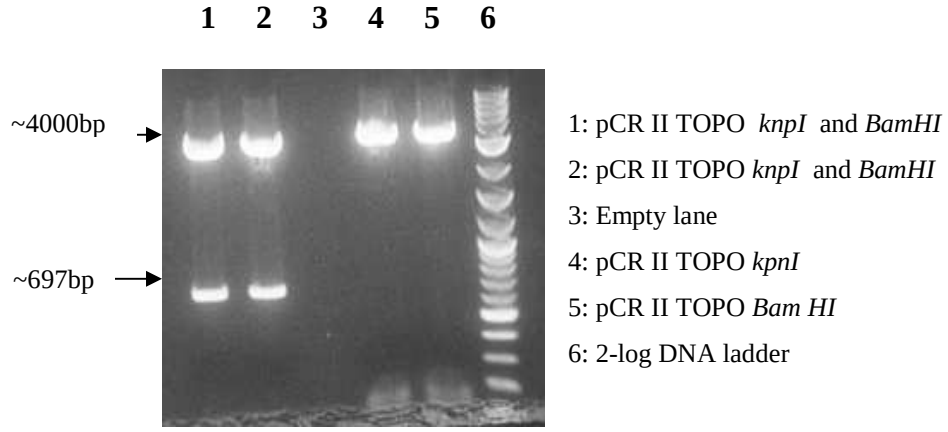
1% agarose gel electrophoresis of PCR amplified products generated with combined CD55 leader-SCR3 primer set. Varying annealing temperatures used to optimise product generation and no non-specific products present in gel analysis. 55°C chosen as optimal annealing temperature for combined primer set.

4.4: Cloning of CD55 leader SCR 1-3 into Signal pIgplus vector

The CD55 leader SCR 1-3 domain was isolated from the PCR II-TOPO vector by *kpnI* and *BamHI* restriction endonuclease digestion. PCR II-TOPO without any insert was digested with both of the enzymes in order to confirm successful digestion of the plasmid. Restriction digest samples were analysed on a 1% agarose gel (**Figure 4.4**). The CD55 leader SCR 1-3 can be seen as a 697bp band and the digested PCR II-TOPO produces a 4kb DNA band.

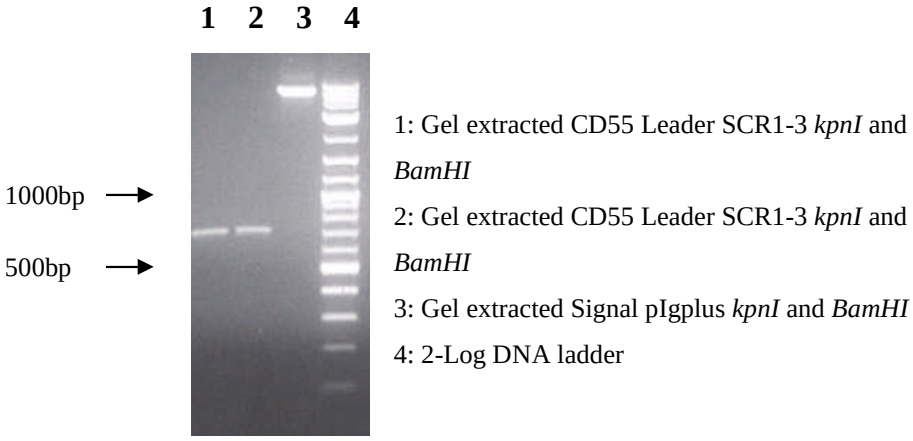
The Signal pIgplus plasmid was also cut with *kpnI* and *BamHI* restriction enzymes and the products ran on a 1% agarose gel. The linear Signal pIgplus and 697bp CD55 leader SCR 1-3 fragments were excised from the gels and phenol/chloroform precipitation was used to clean up the digested products. These digests were then electrophoresed on a 1% agarose gel to confirm that the DNA had not been lost during the clean up procedure (**Figure 4.5**).

Figure 4.4: PCR II TOPO +/- (CD55 leader SCR 1-3) restriction digest with *kpnI* and *BamHI*



1% agarose gel electrophoresis of pCR II Topo CD55 leader SCR 1-3 digested with *kpnI* and *BamHI* restriction endonucleases. Successful removal of CD55 leader SCR 1-3 producing band of ~697bp and linear pCR II TOPO plasmid ~4000bp.

Figure 4.5: Restriction enzyme digest of CD55 leader SCR 1-3 PCR products post purification

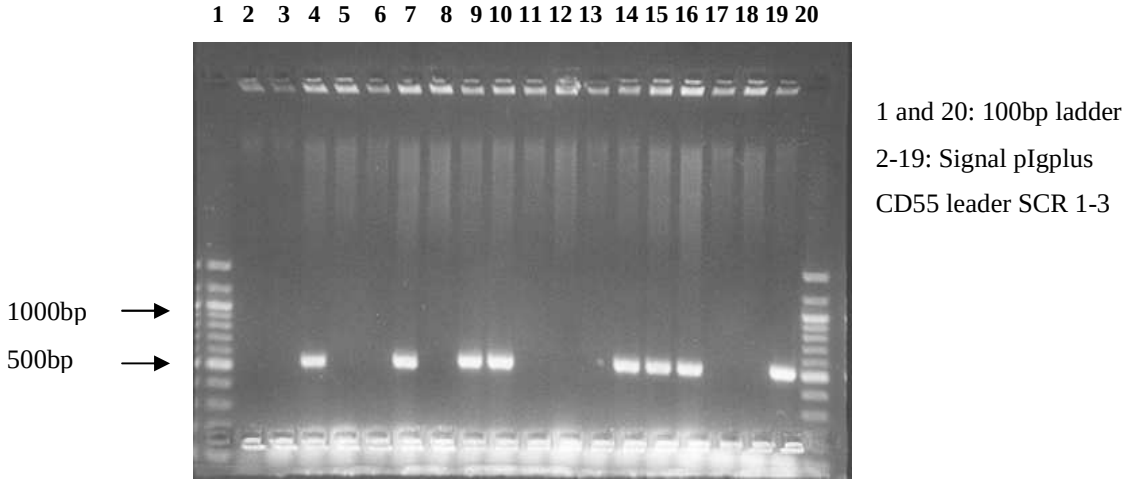


1% agarose gel electrophoresis of restriction endonuclease digested CD55 leader SCR1-3 PCR product and signal pIgplus plasmid. PCR products excised from agarose and phenol/chloroform DNA precipitated. Products digested with both *kpnI* and *BamHI* for ligation into Signal pIgplus plasmid. Double digested PCR products show a band of ~697bp. Restricted plasmid generates a band ~6302bp (removed 39bp of multiple cloning site by enzyme restriction).

The CD55 leader SCR 1-3 product was then ligated utilising T4 DNA ligase into the *kpnI* /*BamHI* sites of the restricted signal pIgplus vector. DH5α competent bacteria were then transformed with the new construct and colonies were screened for the DNA insert via PCR with T7 forward and DSCR23 reverse (binds end of SCR2 of CD55) primers. **Figure 4.6** shows the results of the colony screen with positive

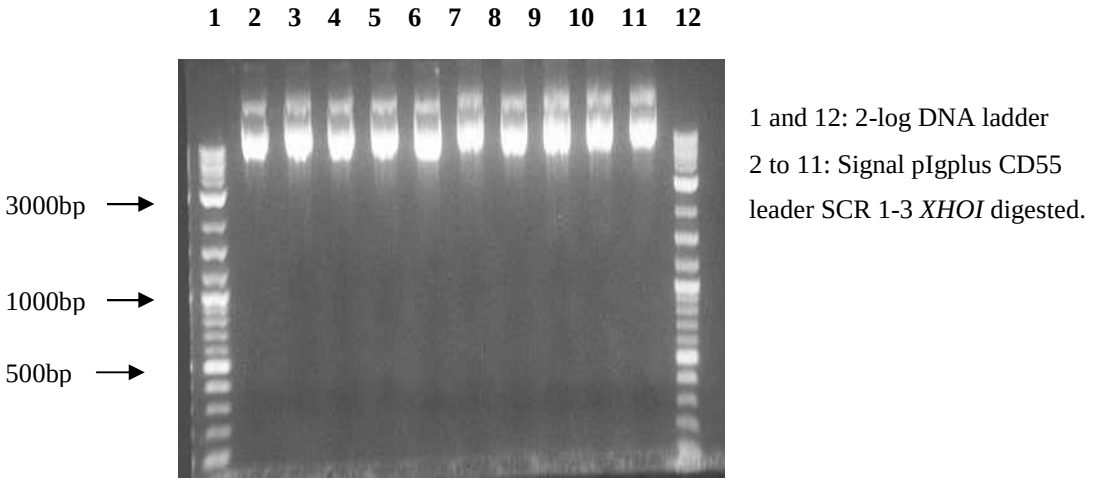
colonies yielding a 541bp fragment. DNA minipreps of positive colonies were carried out and construct integrity was checked by using a negative control restriction digest with *XHOI*. Results are shown in **Figure 4.7** displaying super-coiling of all DNA minipreps as no digestion of the plasmid has occurred.

Figure 4.6: Screen of DH5α colonies transformed with Signal pIgplus containing CD55 leader SCR 1-3. Screened using T7 and DSCR 23 reverse primers.



1% agarose gel electrophoresis of DH5α colonies transformed with Signal pIgplus plasmid encoding CD55 leader SCR1-3. PCR analysis of colonies with T7 forward primer and DSCR 23 reverse primer yielding a product from positive colonies containing ~541bp fragment. DSCR 23 reverse primer designed for amplification of SCR 2 region of CD55, yielding smaller fragment.

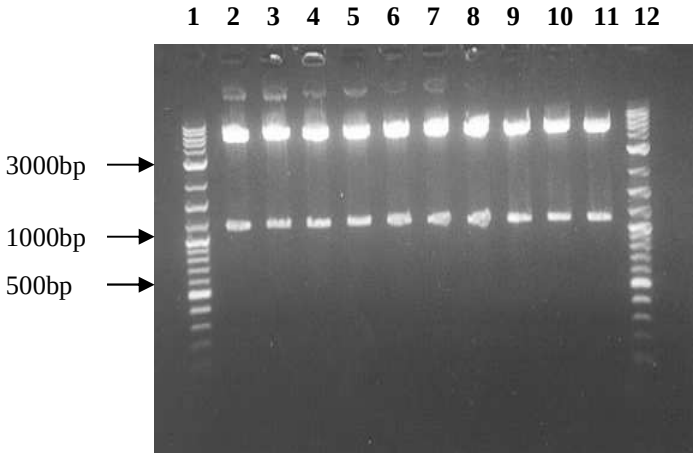
Figure 4.7: Control restriction enzyme digest analysis of minipreps containing CD55 Leader SCR1-3 for assessment of construct integrity



1% agarose gel electrophoresis of Signal pIgplus encoding CD55 leader SCR 1-3 Fc restriction endonuclease digested with *XHOI*. No restriction site should be present in either insert or plasmid allowing supercoiling of construct DNA.

Further assessment of the complete construct was required and therefore digested using *BglII* and *Hind III* restriction enzymes. This double digest should generate two bands one of 1112bp and one of 5740bp. These two enzymes were chosen as *BglII* cuts once within the Signal pIgplus sequence, and *Hind III* cuts once in the CD55 sequence. The results are shown in **Figure 4.8** and clearly show the presence of two bands.

Figure 4.8: Signal pIgplus CD55 leader SCR 1-3 construct restriction digest with *Bgl II* and *Hind III* (assessment of complete construct)



1 and 12: 2-log DNA ladder
2 to 11: Signal pIgplus CD55 leader SCR 1-3 *BglII/Hind III* digested.

1% agarose gel electrophoresis of CD55 leader SCR 1-3 Signal pIgplus construct restriction endonuclease digested with *Hind* III and *BglII* enzymes for assessment of complete construct.
Hind III cuts construct at position 266 of CD55 only.
Bgl II cuts Signal pIgplus at position 5394.
CD55 leader SCR 1-3 (71-740bp of CD55) incorporated into plasmid at *kpnI / BamHI* sites (6311-5357).
2 fragments expected : (6311-5394) + (266-71) = **1112bp**
BglI to start of insert + CD55 to *Hind III*
: (740-771-266) + (5394-57) = **5740bp**
CD55 insert minus 266 cut with *HindIII* + Signal pIgplus minus region cut with *BglII*.
Control digest indicates complete CD55 leader SCR 1-3 has been incorporated into Signal pIgplus plasmid.

4.5: Sequencing of the CD55 leader SCR 1-3 in Signal pIgplus

The CD55 leader SCR 1-3 Signal pIgplus construct was sequenced using the SCR3 reverse primer (**Figure 4.9)**. The returned sequence was run in the NCBI sequence alignment program with the full-length human CD55 sequence. Results show complete alignment of the sequence with no mutations present in the generated construct. The chosen insert is in frame with restriction sites present in the Signal

pIgplus plasmid and the human IgG Fc tail. **Figure 4.10** shows the complete sequence of the Signal pIgplus plasmid encoding for CD55 leader SCR 1-3 in frame with Fc tail.

Figure 4.9: Sequence of CD55 leader SCR1-3 in Signal pIgplus vector using SCR 3 Reverse primer

```
  1NGCTNNGNNG ANGGGACCCC CACCCCCCAC CACCTTCGAT GGGATCCGCT TCTCTGCACT60
 61CTGGCAACGG GTCACTCCAC TGGACAGAGC TGCCTGAAAT AAGACAAAAA CTAGAAGTCG120
121AGCCAAATAA TTTGTACCCT GTGTTACATG AGAAGGAGAT GGTTGCACCA AATAATATGC180
181CACCTGGTAC ATCAATCTGA CCATTTCGTA TTTCTCCCGG ATTAGGGCAT GATTTCTTTT240
241TACAAAATTC GACTGCTGTG GACCATTTTA AATTCTGAAG GCAAGTTAGT TTTGGTGATA300
301GAGAAGGTTC TCTTCTGTAA CCTGGACGGC ACTCATATTC CACAACAGTA CCGACTGGAA360
361AATAATTCTG AGTGATATAA GGCTGTTTGA GGGATGCAGA ATTTAGCCTT GTTGGCACCT420
421CGCAGCTACG ATTGCAGAAC TCTTCAATAT CTGACCATTG ACTGCCCTTA AGGCAGATCA480
481CTGAGTCCTT CTCGCCAGGA ATTTTCACAA AGCTTTCTTC ACATTTGTAC GTTATTACAG540
541TATCCTCGGG AAAACTTGTA CGGCCTTCCA AAGCTGGCTG GGCATTAGGT ACATCTGGGG600
601GAAGGCCACA GTCACCCCAC ACGGCCGGCA GGCACAACAG CACCAGCAGC AGCAGCCGGG660
721GCAGCTCCCC GAGGAGGGGC AGCGCCGCGG GCACGCTCGG CCGCGCGACG GTCATGGCGC720
781GCCGGGTACC AGCTTGGGTC TCCCTATAAG TGAGTCGTAT TAATTTTCGA TAAGCCAGTA780
```

GG SCR 3 Reverse primer

ACC Former factor Xa cleavage site (GCA)

GGGATC *BamHI* site

CG CD55 leader forward primer

GGTACC *kpnI* site cloned into Signal pIgplus plasmid

NCBI alignment of returned sequence with accession number BC001288 [Homo sapiens decay accelerating factor for complement (CD55, Cromer blood group system)]

```
50  ttctctgcactctggcaacgggtcactccactggacagagctgcctgaaataagacaaaa 109
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
745 ttctctgcactctggcaacgggtcactccactggacagagctgcctgaaataagacaaaa 686
203  E  R  C  E  P  L  P  D  S  W  Q  V  S  S  G  S  I  L  C  F

110 actagaagtcgagccaaataatttgtaccctgtgttacatgagaaggagatggttgcacc 169
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
685 actagaagtcgagccaaataatttgtaccctgtgttacatgagaaggagatggttgcacc 626
183  S  S  T  S  G  F  L  K  Y  G  T  N  C  S  F  S  I  T  A  G

170 aaataatatgccacctggtacatcaatctgaccatttcgtatttctcccggattagggca 229
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
625 aaataatatgccacctggtacatcaatctgaccatttcgtatttctcccggattagggca 566
163  F  L  I  G  G  P  V  D  I  Q  G  N  R  I  E  G  P  N  P  C

230 tgatttctttttacaaaattcgactgctgtggaccattttaaattctgaaggcaagttag 289
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
565 tgatttctttttacaaaattcgactgctgtggaccattttaaattctgaaggcaagttag 506
143  S  K  K  K  C  F  E  V  A  T  S  W  K  L  N  Q  L  C  T  L

290 ttttggtgatagagaaggttctcttctgtaacctggacggcactcatattccacaacagt 349
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
505 ttttggtgatagagaaggttctcttctgtaacctggacggcactcatattccacaacagt 446
123  K  P  S  L  S  P  E  R  R  Y  G  P  R  C  E  Y  E  V  V  T

350 accgactggaaaataattctgagtgatataaggctgtttgagggatgcagaatttagcct 409
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
445 accgactggaaaataattctgagtgatataaggctgtttgagggatgcagaatttagcct 386
103  G  V  P  F  Y  N  Q  T  I  Y  P  Q  K  L  S  A  S  N  L  R
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410 tgttggcacctcgcagctacgattgcagaactcttcaatatctgaccattgactgcccttt 469
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
385 tgttggcacctcgcagctacgattgcagaactcttcaatatctgaccattgactgcccttt 326
83   T  P  V  E  C  S  R  N  C  F  E  E  I  D  S  W  Q  S  G  K

470 aaggcagatcactgagtccttctcgccaggaattttcacaaagctttcttcacatttgtat 529
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
325 aaggcagatcactgagtccttctcgccaggaattttcacaaagctttcttcacatttgtat 266
 63   L  C  I  V  S  D  K  E  G  P  I  K  V  F  S  E  E  C  K  Y

530 cgttattacagtatcctcgggaaaacttgtacggccttccaaagctggctgggcattagg 589
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
265 cgttattacagtatcctcgggaaaacttgtacggccttccaaagctggctgggcattagg 206
 43   T  I  V  T  D  E  P  F  S  T  R  G  E  L  A  P  Q  A  N  P

590 tacatctgggggaaggccacagtcaccccacacggccggcaggcacaacagcaccagcag 649
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
205 tacatctgggggaaggccacagtcaccccacacggccggcaggcacaacagcaccagcag 146
23   V  D  P  P  L  G  C  D  G  W  V  A  P  L  C  L  L  V  L  L

650 cagcagccggggcagctccccgaggaggggcagcgccgcgggcacgctcggccgcgcgac 709
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
145 cagcagccggggcagctccccgaggaggggcagcgccgcgggcacgctcggccgcgcgac 86
3    L  L  R  P  L  E  G  L  L  P  L  A  A  P  V  S  P  R  A  V

710 ggtcatggcgcgccgggt 727
    ||||||||||||||||||
85  ggtcatggcgcgccgggt 68
1    T  M
```

No mutations present in CD55 leader SCR 1-3 compared to wild type CD55.
Insert is in-frame with human IgG (Fc tail) of Signal pIgplus plasmid.

Figure 4.10: Sequence of CD55 leader SCR 1-3 Fc in Signal pIgplus plasmid

AAT	T7 Primer	ATG – GAC	CD55 leader sequence
GGT ACC	*kpnI* restriction site		
GAT CCC	*BamHI* restriction site		
GGT	Former factor Xa cleavage site		
KSC	Start of human IgG Fc tail		
TCT	pIgplus SEQ 3' primer		

```
GAA ATT AAT ACG ACT CAC TAT AGG GAG ACC CCA GCT GGT ACC CGG CGC GCC
                                              T   R   R   A
ATG ACC GTC GCG CGG CCG AGC GTG CCC GCG GCG CTG CCC CTC CTC GGG GAG
 M   T   V   A   R   P   S   V   P   A   A   L   P   L   L   G   E
CTG CCC CGG CTG CTG CTG CTG GTG CTG TTG TGC CTG CCG GCC GTG TGG GGT
 L   P   R   L   L   L   L   V   L   L   C   L   P   A   V   W   G
GAC TGT GGC CTT CCC CCA GAT GTA CCT AAT GCC CAG CCA GCT TTG GAA GGC
 D   C   G   L   P   P   D   V   P   N   A   Q   P   A   L   E   G
CGT ACA AGT TTT CCC GAG GAT ACT GTA ATA ACG TAC AAA TGT GAA GAA AGC
 R   T   S   F   P   E   D   T   V   I   T   Y   K   C   E   E   S
TTT GTG AAA ATT CCT GGC GAG AAG GAC TCA GTG ATC TGC CTT AAG GGC AGT
 F   V   K   I   P   G   E   K   D   S   V   I   C   L   K   G   S
CAA TGG TCA GAT ATT GAA GAG TTC TGC AAT CGT AGC TGC GAG GTG CCA ACA
 Q   W   S   D   I   E   E   F   C   N   R   S   C   E   V   P   T
AGG CTA AAT TCT GCA TCC CTC AAA CAG CCT TAT ATC ACT CAG AAT TAT TTT
 R   L   N   S   A   S   L   K   Q   P   Y   I   T   Q   N   Y   F
CCA GTC GGT ACT GTT GTG GAA TAT GAG TGC CGT CCA GGT TAC AGA AGA GAA
 P   V   G   T   V   V   E   Y   E   C   R   P   G   Y   R   R   E
CCT TCT CTA TCA CCA AAA CTA ACT TGC CTT CAG AAT TTA AAA TGG TCC ACA
 P   S   L   S   P   K   L   T   C   L   Q   N   L   K   W   S   T
GCA GTC GAA TTT TGT AAA AAG AAA TCA TGC CCT AAT CCG GGA GAA ATA CGA
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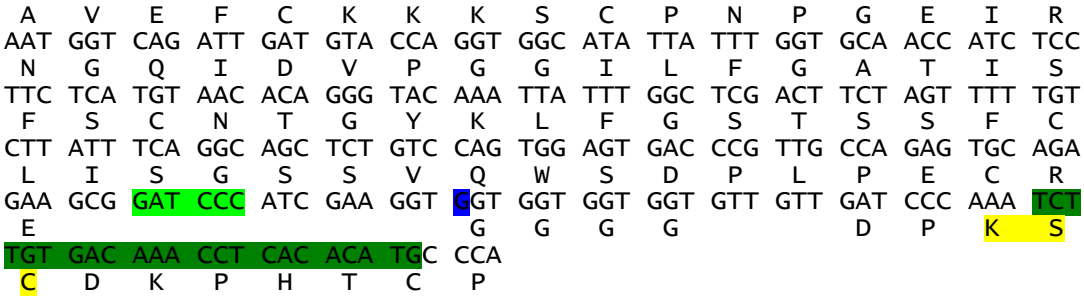


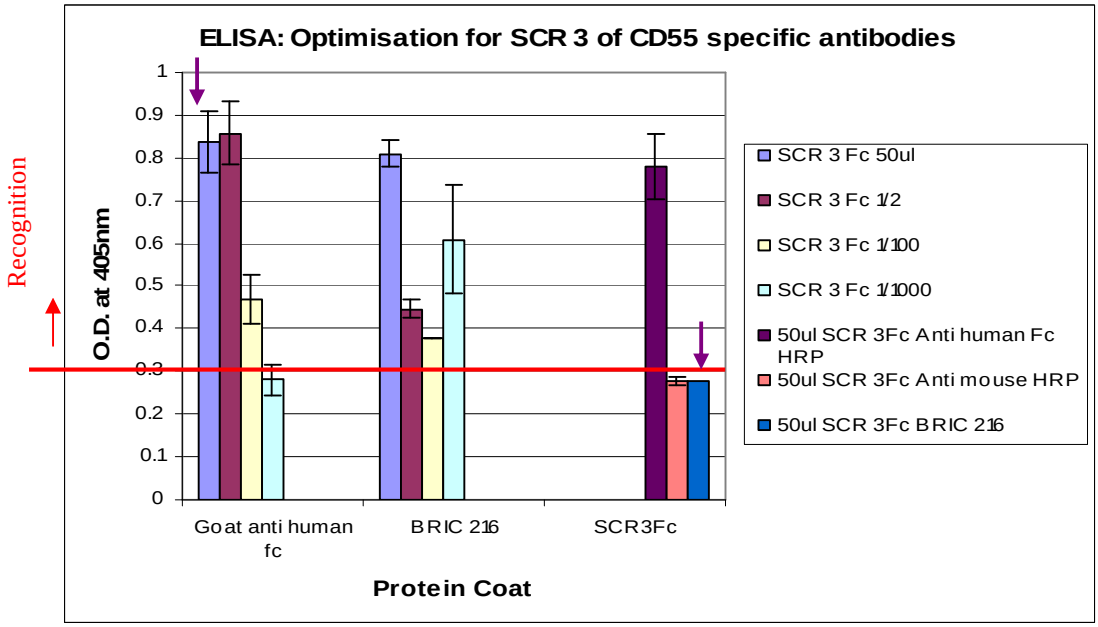
4.6: Eukaryotic expression of SCR 1-3Fc fusion protein

CHO cells obtained for viability within serum free media were selected as suitable targets for transfection with SCR 1-3 Fc DNA construct for expression of CD55 fusion protein. Serum free conditions were preferable in order to obtain pure fusion protein containing no contaminating serum proteins. Transfection of target cells was carried out using DMRIE C reagent and 2µg, 4µg and 6µg SCR1-3 Fc DNA. Selective pressure was applied for culture of successfully transfected targets with 500µg/ml G418 sulphate. (As assessed with antibiotic kill curve of non transfected CHO-SF cells). Transfected cells were cultured for two weeks maintaining selection pressure and supernatant was collected. Purification of expressed protein was carried out using protein G columns in order to selectively bind fusion protein mediated via human Ig Fc tail. Protein was assessed via anti human Fc capture ELISA determining viable expression of CD55 domains as assessed via CD55 specific antibodies.

A protein assay was initially set up for analysis of protein production and presence within culture supernatant. The assay was used with SCR3 Fc fusion protein, which was previously generated following identical methodology as per the SCR 1-3 Fc construct. Initial ELISA analysis using direct binding of purified protein or culture supernatant to the plate appeared to prevent specific antibody recognition of SCR 3 domain of CD55, while anti Fc HRP conjugated antibodies were successful at binding the Fc region of the protein. It was hypothesised that either due to innate charge or size of the protein and its domains that the SCR 3 region was being 'masked' either by the Fc domain or due to orientation upon the plate surface. The alternative was that the fusion protein was not being expressed as hoped with altered post translational modifications, making the SCR 3 domain non-functional. In order to assess this, a capture protocol was designed using a goat anti human IgG (Fc specific) monoclonal antibody as the primary coat. This enabled correct orientation of the Fc fusion protein allowing exposure of the active CD55 component. **Figure 4.11** shows

the optimised protocol for protein assessment. Titrating concentrations of protein were bound to anti human Fc and SCR3 presence was identified using BRIC 216 antibody (SCR3 specific). This was compared to direct binding of fusion protein or capture via BRIC 216 coated wells. Results clearly show that with Fc capture of recombinant protein, the SCR 3 domain is clearly recognised by relative anti CD55 antibodies, compared to assay procedure without capture preventing BRIC 216 recognition of its target.

Figure 4.11: ELISA analysis of SCR 3 Fc fusion protein screen indicating the necessity for correct orientation of recombinant protein on the ELISA plate. Optimisation of fusion protein screen



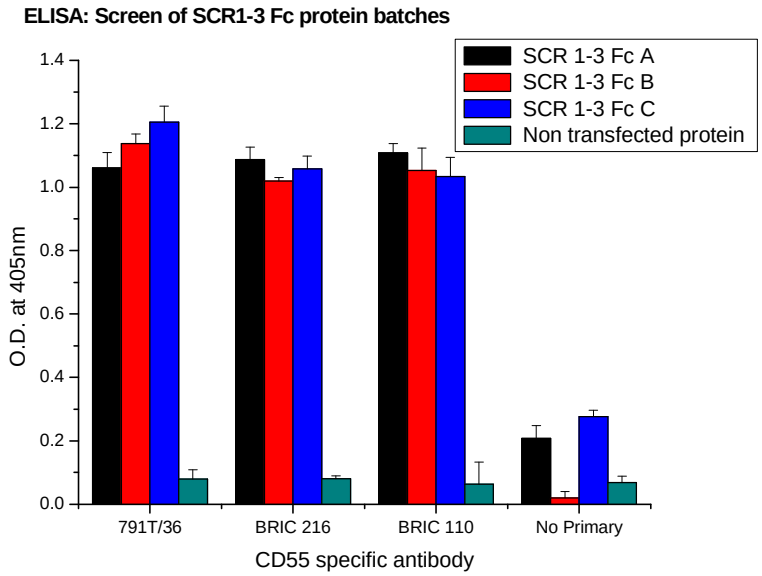
→ Comparison between Fc captured SCR 3 Fc protein compared to direct binding of protein to plate without orientation.

96 well flat bottom flexi plates coated with 100µl of 5µg/ml goat anti human IgG (Fc specific) antibody, BRIC 216 (SCR3 Fc specific) and SCR 3Fc recombinant protein. SCR 3 Fc titrations (1mg/ml stock) applied to coating antibodies in order to assess if protein orientation affected ability of secondary antibody recognition when compared to fusion protein as primary coat. Anti-human Fc captured protein was assessed with BRIC 216 (CD55 specific) and horse radish peroxidase conjugated rabbit anti-mouse antibody. BRIC 216 captured fusion protein was assessed directly with goat anti-human Fc HRP conjugated antibody. ABTS was used as a substrate and plate were read at 405nm. Error bars indicate standard deviation for two data points.

Figure 4.12 shows analysis of SCR 1-3 Fc fusion protein obtained from transfected CHO-SF cells. The fusion protein was captured with anti human Fc specific

antibodies and the presence of functional CD55 domains was assessed by 791T/36 (SCR1-2), BRIC 16 (SCR3) and BRIC 110 (SCR2) monoclonal antibodies. All three protein batches, from separate transfection protocols, bound to all 3 CD55 specific antibodies indicating the presence of SCR 1-3 fused to a human IgG Fc domain protein. This could then be used for both direct immunisation and for screening of any serum antibodies generated in response to the SCR 1-3 (wild type) Fc DNA construct vaccine.

Figure 4.12: ELISA of SCR 1-3 Fc fusion protein batches



A = 2µg SCR 1-3 Fc DNA

B = 4µg SCR 1-3 Fc DNA

C = 6µg SCR 1-3 Fc DNA

791T/36 specific to human CD55 SCR domains1-2

BRIC 216 specific to human CD55 SCR domain 3

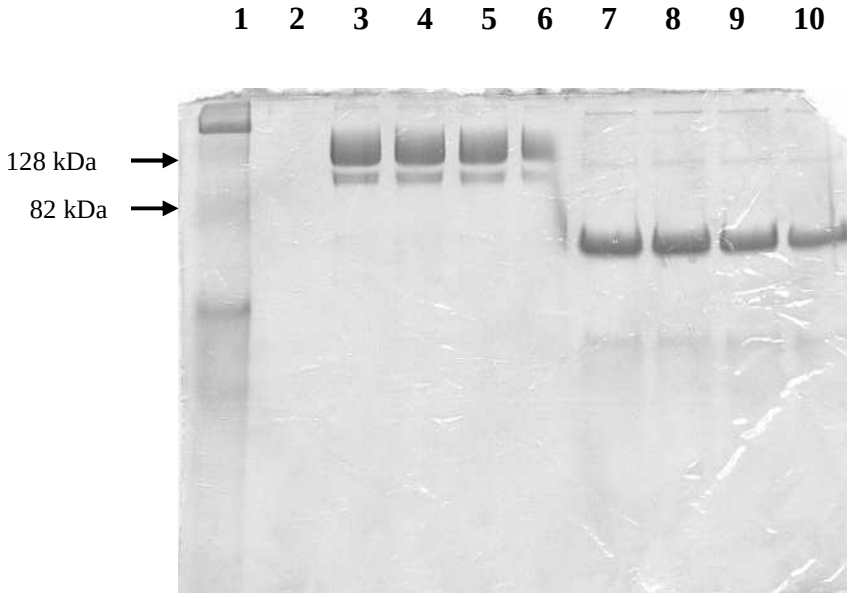
BRIC 110 specific to human CD555 SCR domain 2

96 well flat bottom flexi plate coated with 100µl of 5µg/ml goat anti human Fc capture antibody and 100µl of 5µg/ml SCR 1-3 Fc protein. Equal concentrations of CD55 specific antibodies were then incubated at a concentration of 5µg/ml (100µl) and secondary rabbit anti-mouse peroxidase conjugated antibodies were added. ABTS was used as a substrate and plates were read at 405nm. Error bars indicate standard deviation for two data points.
All batches of fusion protein contain structurally complete and native CD55 domains.

SCR1-3 Fc protein was also assessed via SDS PAGE (**Figure 4.13)** and western blot (**Figure 4.14)** for determination of purity and functionality. 10% SDS PAGE analysis under non-reducing conditions generates a double band, indicating several

glycosylation states of expressed protein, approximately 100kDa in size representing SCR1-3 Fc domain. No other proteins are visible within the samples indicating a high degree of purity from purification procedure. Breaking of disulphide bonds under reducing conditions produces an equivalent band of ~70kDa. Western blot analysis indicates that both 791T/36 and BRIC 216 bind to the SCR 1-3 domain of the fusion protein yielding a band of ~100kDa. Antibodies specific for BSA generated no bands and, as expected, none of the CD55 specific antibodies were able to bind target domains under reducing conditions (prior analysis has shown under reducing conditions the CD55 structure is significantly altered to prevent antibody recognition).

Figure 4.13: 10% SDS PAGE analysis of SCR 1-3 Fc recombinant protein under reducing and non-reducing conditions.

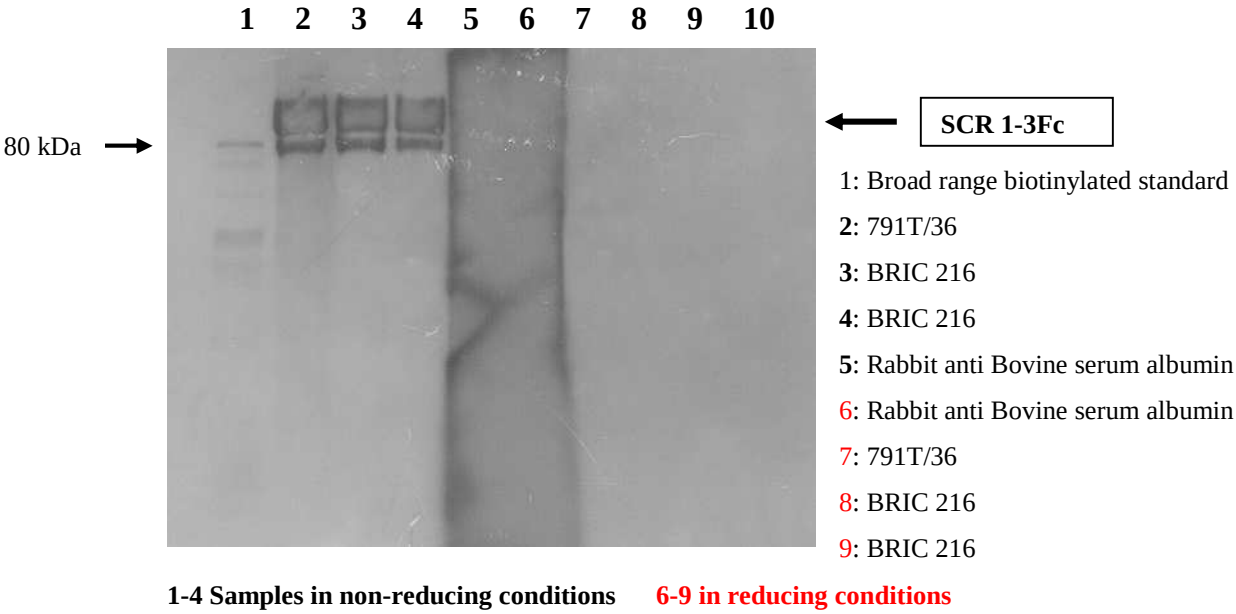


1: Broad range kaleidoscope markers
2: Broad range biotinylated standard
3: SCR 1-3 Fc protein
4: SCR 1-3 Fc protein
5: SCR 1-3 Fc protein
6: SCR 1-3 Fc protein
7: SCR 1-3 Fc protein
8: SCR 1-3 Fc protein
9: SCR 1-3 Fc protein
10: SCR 1-3 Fc protein

3-6 Samples in non-reducing conditions **7-10 in reducing conditions**

2.4µg of protein were added per well in a 1: 3 ratio with reducing or non- reducing sample buffer. Samples were electrophoresed for 1 hour at 200 volts and the separating gel was visualised with coomassie staining of all proteins present. Under non reducing condition SCR 1-3 Fc produces a double band at ~100kDa representing SCR 1-3 Fc component of the recombined protein with no other bands present within the sample indicating that the protein samples remains pure. The second visualised band represents alternate glycosylation products of the expressed protein. Under reducing conditions, a band of ~70kDa is generated representing the protein with broken disulphide bonds.

Figure 4.14: Western blot analysis of SCR1-3 Fc recombinant protein under reducing and non-reducing conditions.



1-4 Samples in non-reducing conditions **6-9 in reducing conditions**

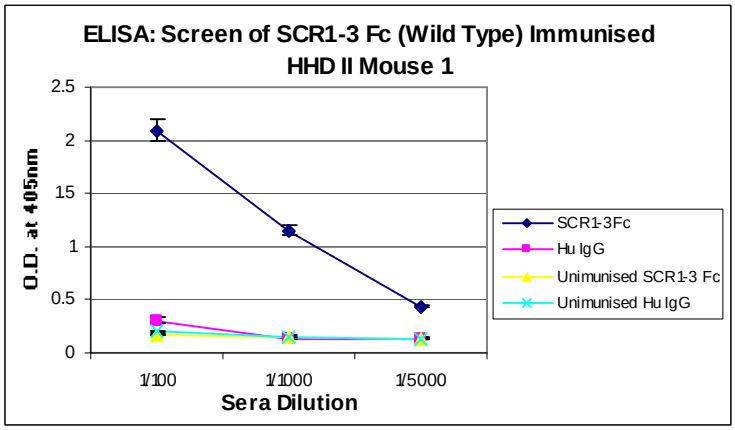
Western blot analysis of figure 2.13. Proteins transferred form acrylamide gel to nitrocellulose membrane and blot was probed with CD55 and BSA specific antibodies. Secondary rabbit anti mouse HRP conjugated antibody was used in conjunction with ECL chemi-luminescence detection system in order to visualise bands on photo sensitive film. All antibodies are specific to native proteins and fail to bind reduced targets. Both 791T/36 and BRIC 216 generate a band ~100kDa in size representing SCR 1-3 Fc protein under non-reducing conditions. Previous assessment of both CD55 specific antibodies has shown that under reducing conditions, both antibodies fail to recognise CD55 both as purified protein ands as natively expressed CD55 on 791T osteosarcoma cell lysates.

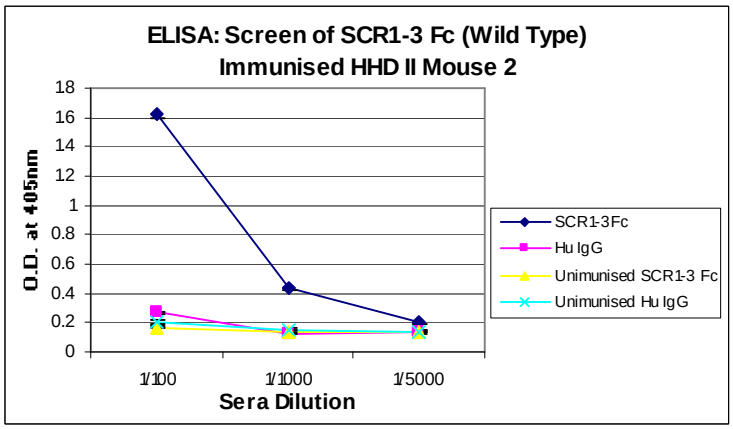
4.7: Antibody response to gene gun delivery of SCR 1-3 (WT) Fc DNA vaccine

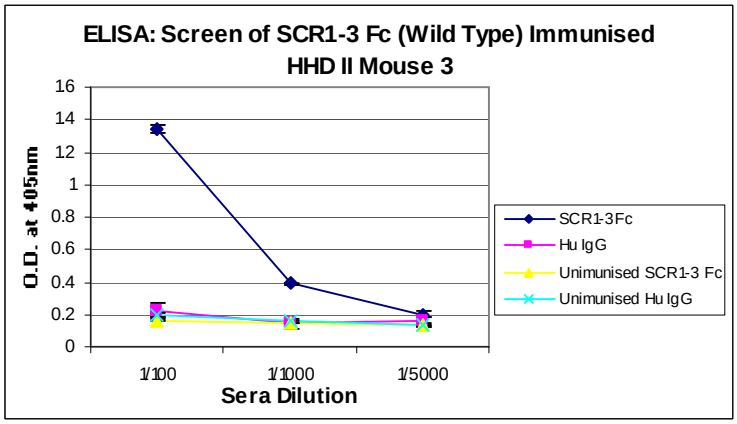
Antibody responses generated following prime boost immunisation of HHD II transgenic mice with Helios gene gun delivery were investigated and titres determined (**Figure 4.15**). 1µg DNA was coated onto 1 micron gold micro-carriers and used to immunise the shaved abdomens of HHD II mice. Immunisations were given at weekly intervals a total of three times, and one week post final vaccination sera were collected to measure the degree of antibody responses raised. Titrated mouse sera were screened against SCR 1-3 Fc recombinant protein and human IgG monoclonal antibodies. Immunised sera were directly compared to responses from naïve serum from un-immunised mice. All four mice in this initial population raised CD55 specific antibodies to a titre greater than 1 in 10^3. Human IgG responses were minimal when compared to un-immunised sera responses to both target proteins.

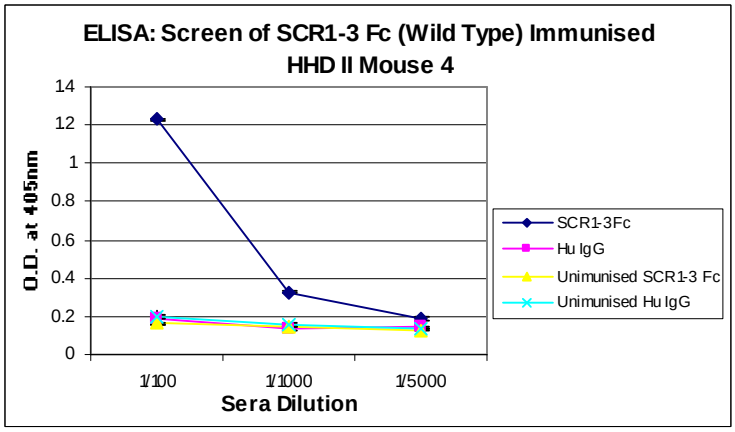
Figure 4.15: ELISA of sera from SCR1-3 Fc DNA (Wild type) Immunised HHD II mice (Group 1)

Anti-CD55 specific antibody responses generated from HHDII mice immunised with heterologous 1µg SCR1-3 Fc DNA, three times at weekly intervals. 1µg of DNA coated onto 1 micron golf micro-particles and immunised via gene gun onto the abdomen of HHD II transgenic mice. Sera obtained one week post final immunisation. Sera titrations screened via ELISA against 5µg/ml SCR1-3 Fc fusion protein and 5µg/ml Human IgG control. Secondary horse radish peroxidase conjugated goat anti mouse IgG antibody was used with ABTS substrate system and absorbance measured at 405nm. Standard deviation expressed as range of two data points.







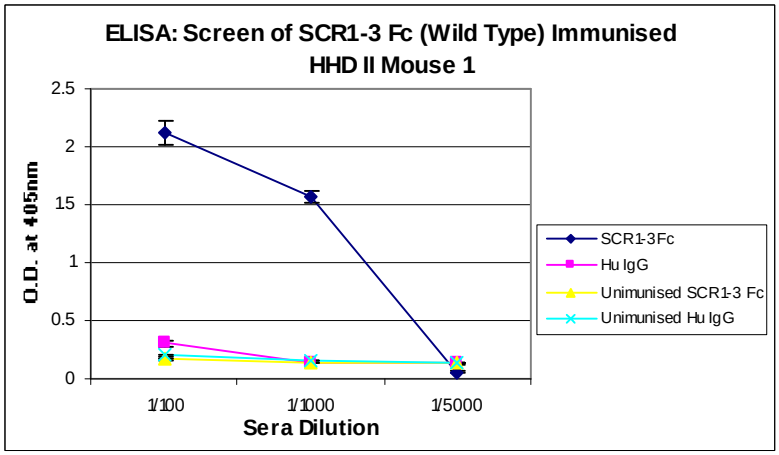


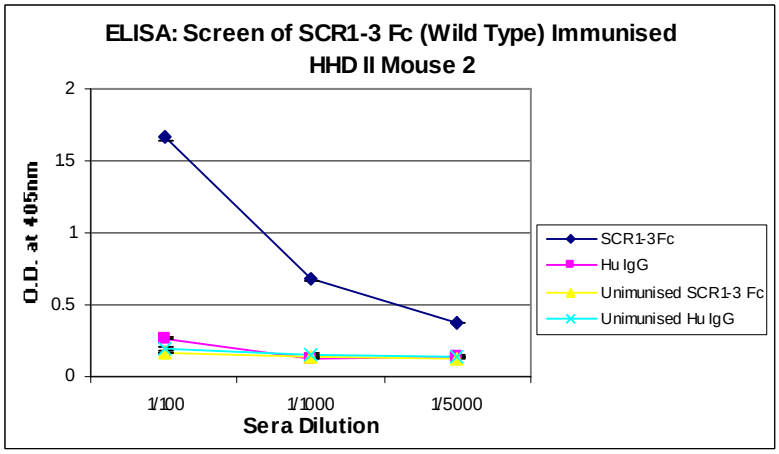
Screen indicates high level of CD55 specificity when compared to human IgG control, indicating minimal response generated to human IgG region of immunising construct. All four mice generate CD55 specific antibodies to a titre greater than 1/1000.

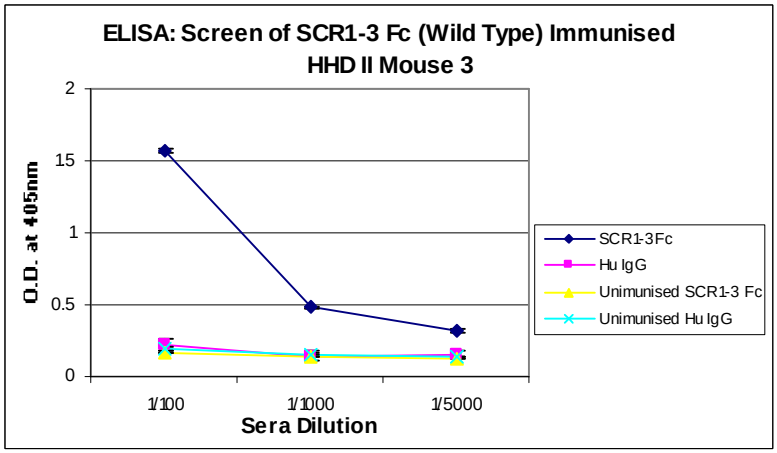
A second group of HHD II transgenic mice were immunised with the wild type construct in order to confirm findings. All four mice in this population raised CD55 specific antibodies to a titre greater than 1 in 10^3 (**Figure 4.16**). Human IgG responses were again minimal when compared to un-immunised sera responses to both target proteins.

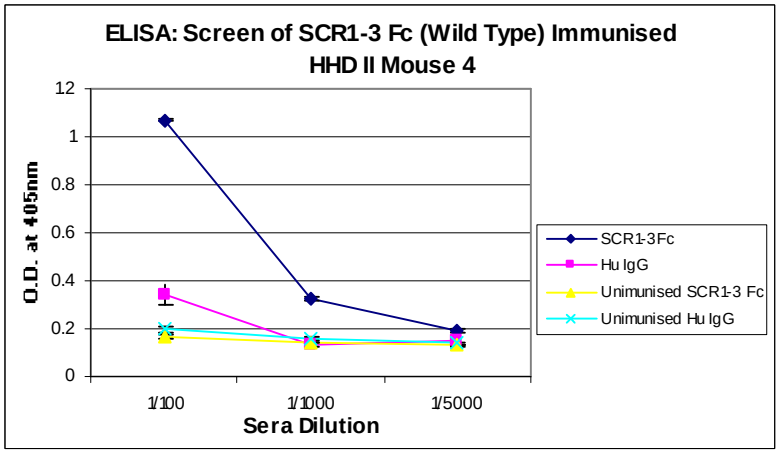
Figure 4.16: ELISA of sera from Group 2 SCR1-3 Fc DNA (Wild type) Immunised HHD II mice (Group 2)

Anti-CD55 specific antibody responses generated from HHDII mice immunised with heterologous 1µg SCR1-3 DNA, three times at weekly intervals. 1µg of DNA coated onto 1 micron golf micro-particles and immunised via gene gun onto the abdomen of HHD II transgenic mice. Sera obtained one week post final immunisation. Sera titrations screened via ELISA against 5µg/ml SCR1-3 Fc fusion protein and 5µg/ml Human IgG control. Secondary horse radish peroxidase conjugated goat anti mouse IgG antibody was used with ABTS substrate system and absorbance measured at 405nm. Standard deviation expressed as range of two data points.









Screen indicates high level of CD55 specificity when compared to human IgG control, indicating minimal response generated to human IgG region of immunising construct. All four mice generate CD55specific antibodies to a titre greater than 1/1000.

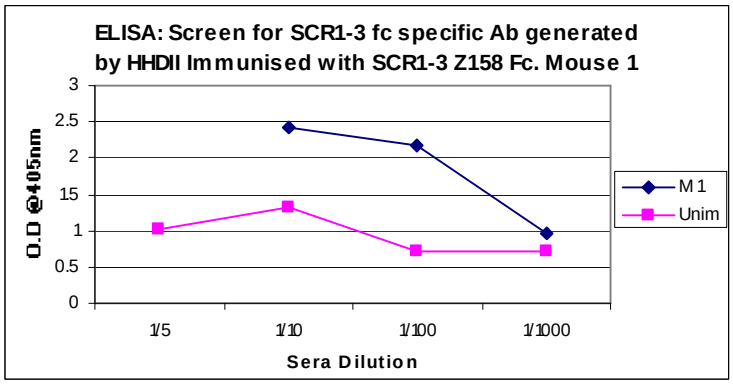
These results indicate that if the Fc domain of the expressed vaccine does indeed enhance processing or delivery of antigen uptake *In vivo*, minimal antibody response is generated in this mouse model to the human IgG (Fc) antigen.

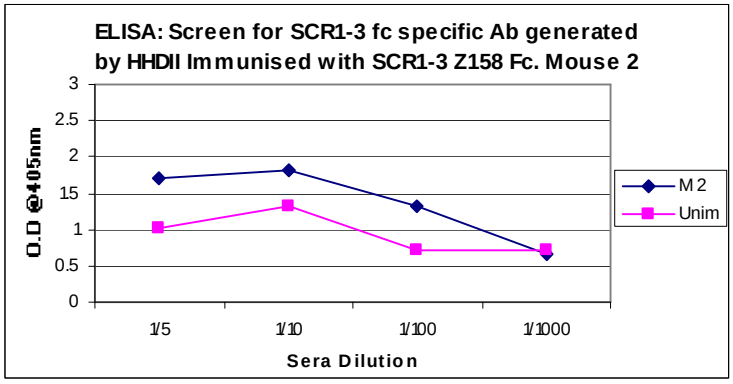
4.8: Antibody response to gene gun delivery of SCR 1-3 Z158m Fc DNA vaccine

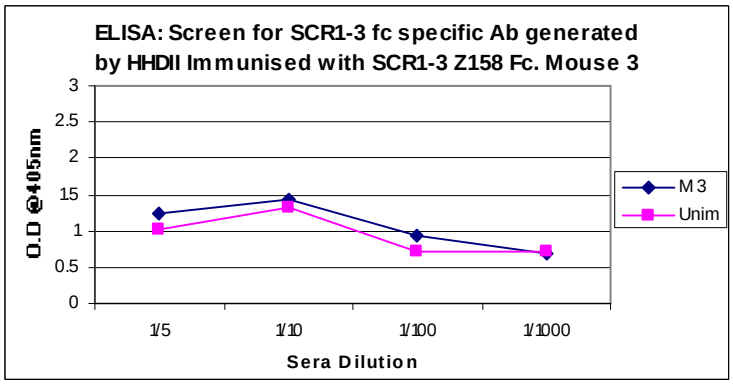
Antibody responses were obtained for the SCR1-3 Fc construct containing the CTL epitope Z158m (**Chapter 5**). Comparisons are made between the two vaccines in order to determine if heteroclitic modification of the wild type construct influences the overall nature of response generated. Immunised sera were directly compared to responses from naïve serum from un-immunised mice. Two of the four mice assessed in the initial group raised antibody mediated responses up to a titre of 1 in 10^3 (**Figure 4.17**). Due to limiting volumes of sera only Mouse 1 from this group was screened for cross reactivity with human IgG, and results indicate that a limited response was specific to this antigen when compared with serum from an un-immunised mouse.

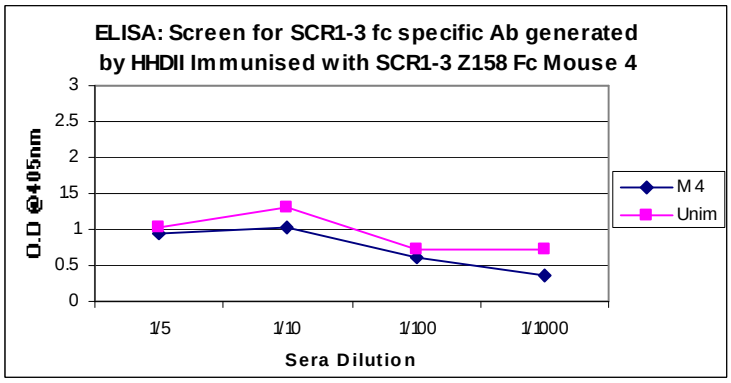
Figure 4.17: ELISA of sera from SCR 1-3 Z158m Fc DNA immunised HHD II mice (Group 1)

Anti CD55 specific antibody responses generated from HHDII mice immunised with heterologous 1µg SCR1-3 Z158 Fc DNA, three times at weekly intervals. 1µg of DNA coated onto 1 micron golf micro-particles and immunised via gene gun onto the abdomen of HHD II transgenic mice. Sera obtained one week post final immunisation. Sera titrations screened via ELISA against 5µg/ml SCR1-3 Fc fusion protein and 5µg/ml Human IgG control. Secondary horse radish peroxidase conjugated goat anti mouse IgG antibody was used with ABTS substrate system and absorbance measured at 405nm. Standard deviation expressed as range of two data points.





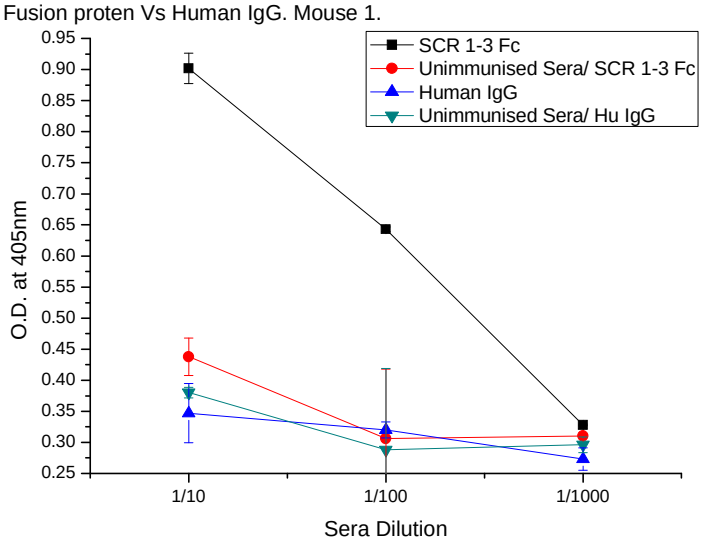




Two out of four mice show antibody responses specific to the SCR 1-3 Fc fusion protein up to a titre of 1/1000 when compared to analysis of un-immunised sera.

Due to limiting volumes of sera obtained from immunised mice only sera from mouse 1 was screened for CD55 SCR 1-3 specificity compared to human IgG.
Sera titration analysed via ELISA against 5µg/ml of either SCR 1-3 Fc protein or human IgG. Rabbit anti mouse HRP conjugated antibodies were used with the ABTS substrate system to evaluate responses. Absorbance was read at 405nm and standard deviation is expressed as two data points.

ELISA: SCR 1-3 Z158 Immunised HHDII sera screen against SCR 1-3 Fc Fusion proten Vs Human IgG. Mouse 1.



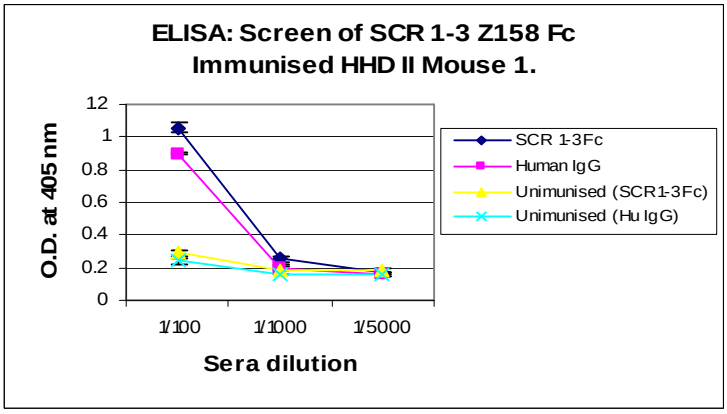
Mouse 1 shows a SCR1-3 specific response when compared to human IgG indicating that the Fc domain of the expressed construct does not appear to drive an overwhelming antibody response.

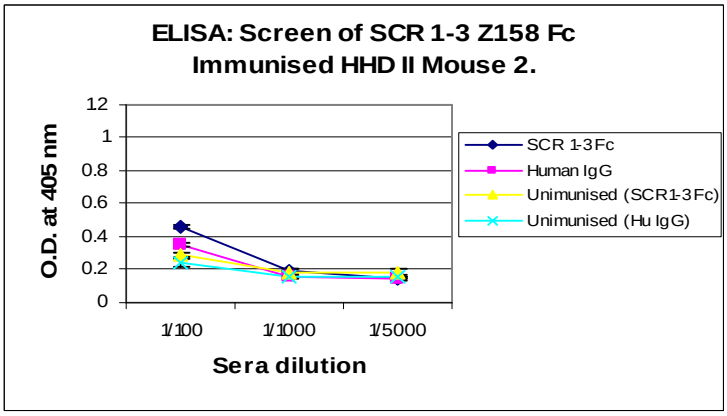
A second group of HHD II mice were immunised with this construct and sera assessment showed significantly different results as compared to the previous group (**Figure 4.18**). Of the four mice screened, antibodies were present at titres of up to 1 in 10^3, although the specificity of the response elicited appears to be mixed as both SCR 1-3 responses and human IgG responses were comparable.

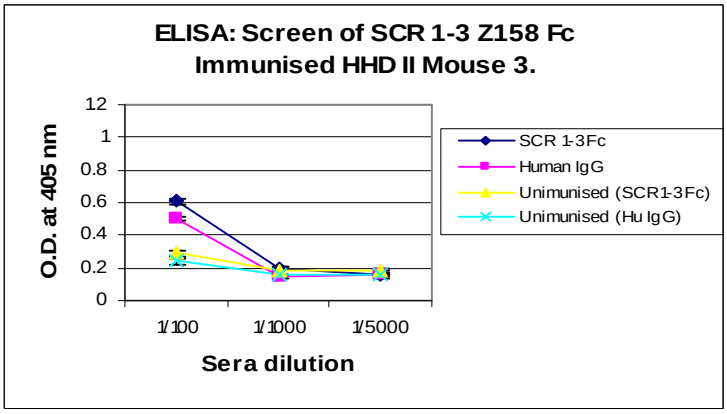
These results could suggest that the mutated vaccine generates a limited CD55 specific antibody response.

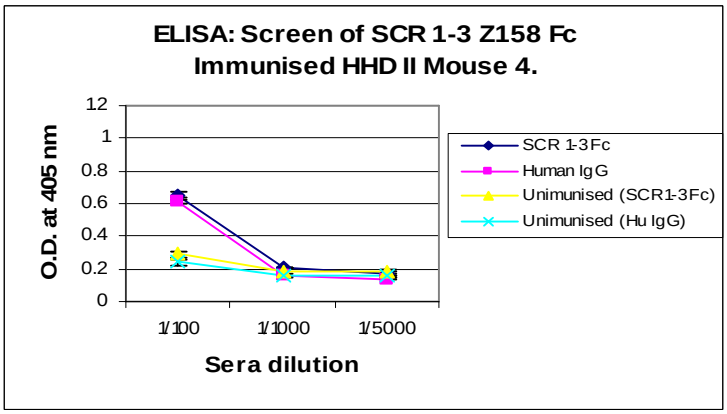
Figure 4.18: ELISA of sera from SCR 1-3 Z158m Fc DNA immunised HHD II mice (Group 2)

Anti CD55 specific antibody responses generated from HHDII mice immunised with heterologous 1µg SCR1-3 Z158 Fc DNA, three times at weekly intervals. 1µg of DNA coated onto 1 micron golf micro-particles and immunised via gene gun onto the abdomen of HHD II transgenic mice. Sera obtained one week post final immunisation. Sera titrations screened via ELISA against 5µg/ml SCR1-3 Fc fusion protein and 5µg/ml Human IgG control. Secondary horse radish peroxidase conjugated goat anti mouse IgG antibody was used with ABTS substrate system and absorbance measured at 405nm. Standard deviation expressed as range of two data points.









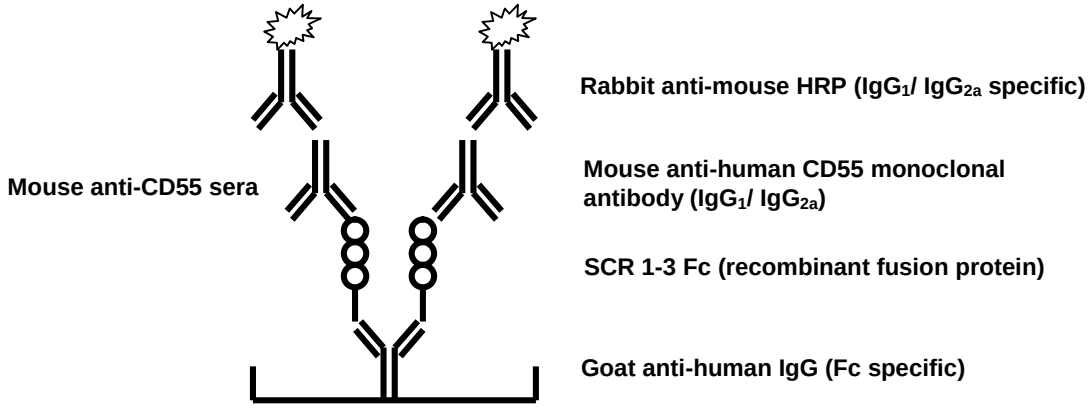
In this population, antibody specificity appears to be reduced with high levels of human IgG Fc specific antibodies being present. Antibody titres are present to titres of 1/1000.

4.9: Antibody Isotyping following gene gun delivery of SCR 1-3 Fc (WT) and Z158m Fc DNA vaccines

Following the immunisation protocol, isotyping of antibody responses was useful in determining the overall nature of the immune response generated. Standard curves were set up using the standard capture ELISA protocol in order that analysis remained consistent with any antibody screen assessment (**Figure 4.19a)**. SCR 1-3 Fc protein was captured and orientated via the human IgG domain using goat anti human Fc

antibodies. IgG_1 and IgG_{2a} CD55 specific antibodies were then bound to the CD55 domains of the protein, titrated to produce an effective logarithmic scale. Peroxidase conjugated rabbit anti mouse antibodies were then used for detection of bound antibodies. The ABTS substrate system was then used to quantify the level of bound proteins. This standard assay was developed for direct comparison with antibody screens performed.

Schematic of antibody isotyping protocol



SCR 1-3 (WT) Fc DNA

All four mice produce predominantly IgG_1 type antibodies, which are clearly visible at both the 1/100 and 1/1000 dilutions (**Figure 4.19b**). Comparison of isotype ratios in all mice (**Figure 4.19c**) show that IgG_{2a} antibody levels are between 3 and 4 $\log^{10}$ lower than IgG_1 antibodies.

Figures 4.20a-c show the repeat assessment of the second group of HHD II mice immunised with this wild type construct. All mice again show a predominantly IgG_1 antibody response up to the 1 in 10^3 dilution and IgG_{2a} antibody levels are between 3 and 4 $\log^{10}$ lower than IgG_1 antibodies.

These results indicate that a potent Th2 response is being driven by the SCR 1-3 Fc DNA vaccine.

Figure 4.19: Isotyping of antibody responses generated by SCR 1-3 (WT) Fc (Group 1)

Figure 4.19a: Standard curve for CD55 specific isotyped antibodies

Standard curve for mouse anti-CD55 specific antibodies was set up using Fc captured SCR 1-3 Fc and titrated concentrations of CD55 specific antibodies BRIC 216 (IgG_1) and IA10 clone (IgG_{2a}) bound to 96 well ELISA plates.

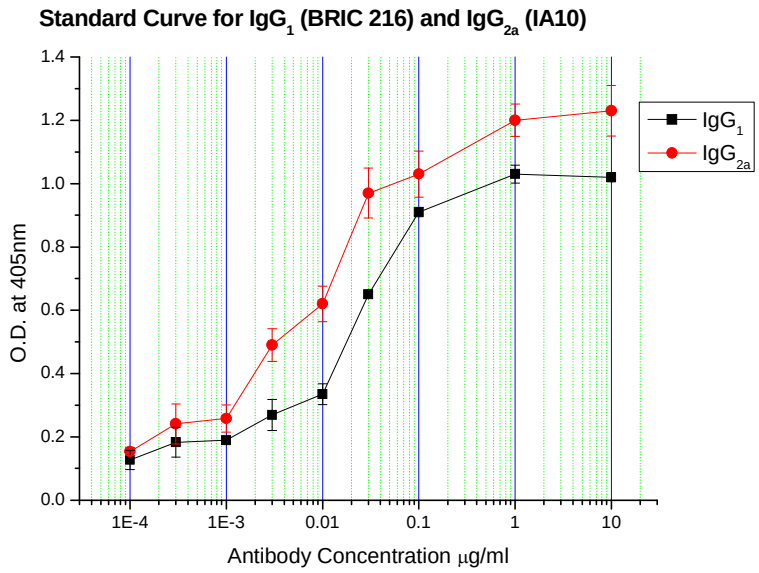
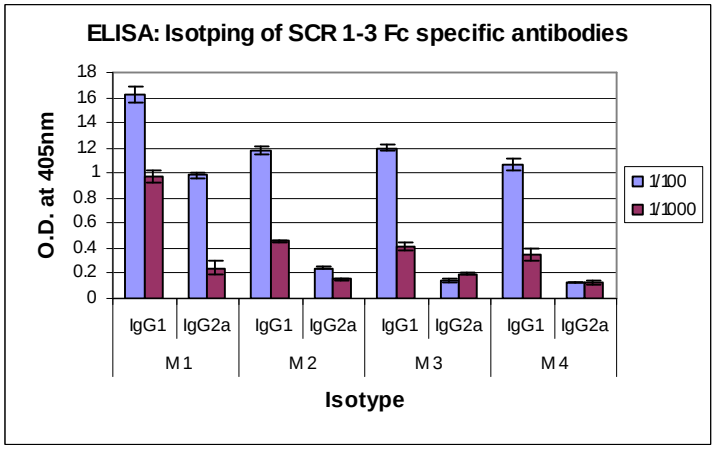


Figure 4.19b: Concentration of isotyped antibody responses



CD55 isotyped responses determined via ELISA assessment of SCR 1-3 Fc DNA immunised mouse sera. Titrated responses observed against SCR 1-3 Fc protein and utilising horse radish peroxidase conjugated goat anti mouse IgG_1 and IgG_{2a} antibodies with ABTS substrate system, measuring absorbance at 405nm. Standard deviation expressed as range of two data points.

Figure 4.19c: Antibody concentrations extrapolated from standard curve and antibody screen results

	IgG_1		IgG_{2a}	
	1/100	**1/1000**	**1/100**	**1/1000**
M1	>10µg/ml	0.12µg/ml	0.012µg/ml	0.001µg/ml
M2	>10µg/ml	0.015µg/ml	0.001µg/ml	~0.0001µg/ml
M3	>10µg/ml	0.012µg/ml	~0.0001µgml	~0.0001µg/ml
M4	0.15µg/ml	0.01µg/ml	~0.0001µg/ml	<0.0001µg/ml

SCR 1-3 Fc (WT) immunisation of HHD II mice drives a predominantly Th2 response producing high titres of IgG_1 antibodies compared with IgG_{2a} levels that are between 3 and 4 log^{10} lower, these results are observed in all four mice assessed in this population.

Figure 4.20: Isotyping of antibody response generated by SCR 1-3 (WT) Fc (Group 2)

Figure 4.20a: Standard curve for CD55 specific isotyped antibodies

Standard curve for mouse anti-CD55 specific antibodies was set up using Fc captured SCR 1-3 Fc and titrated concentrations of CD55 specific antibodies BRIC 216 (IgG_1) and IA10 clone (IgG_{2a}) bound to 96 well plates.

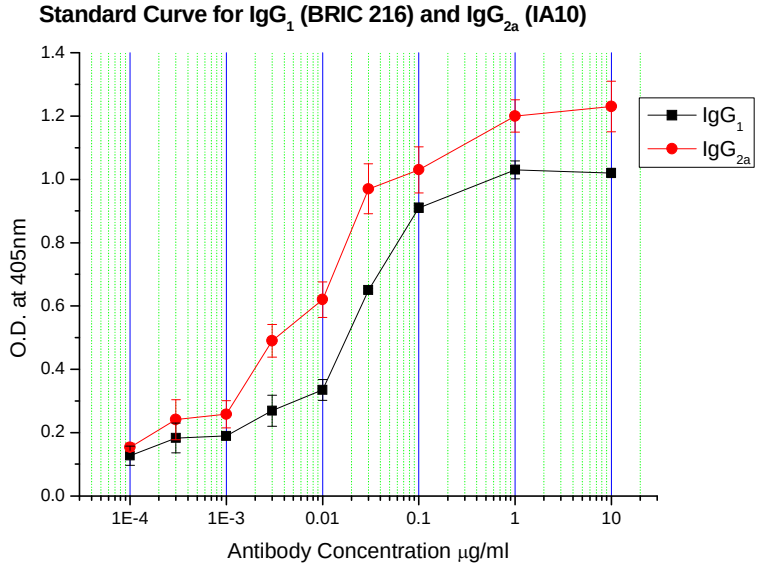


Figure 4.20b: Concentration of isotyped antibody responses

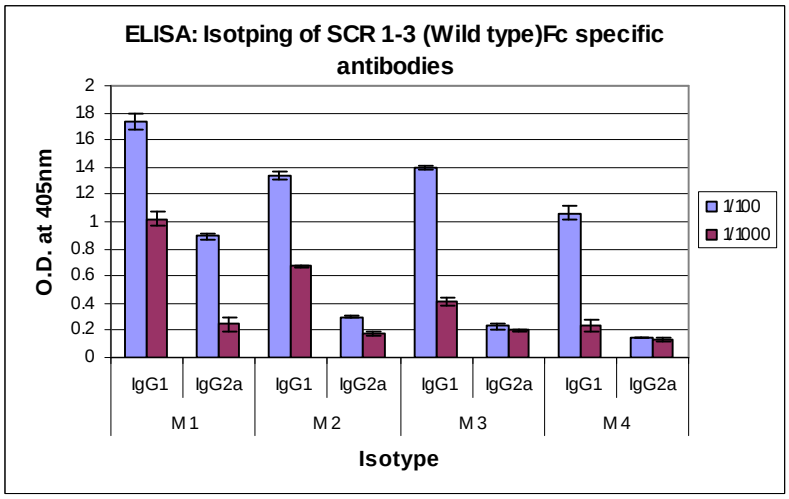


Figure 4.20c: Antibody concentrations extrapolated from standard curve and antibody screen results

	IgG_1		IgG_{2a}	
	1/100	**1/1000**	**1/100**	**1/1000**
M1	>10µg/ml	01-10µg/ml	0.02µg/ml	0.0003µg/ml
M2	>10µg/ml	0.03µg/ml	0.001µg/ml	~0.0001µg/ml
M3	>10µg/ml	0.02µg/ml	~0.0001µgml	~0.0001µg/ml
M4	>10µg/ml	0.001µg/ml	~0.0001µg/ml	<0.0001µg/ml

SCR 1-3 Fc (WT) immunisation of HHD II mice drives a predominantly Th2 response producing high titres of IgG_1 antibodies compared with IgG_{2a} levels that are between 3 and 4 log^{10} lower, these results are observed in all four mice assessed in this population.

SCR 1-3 Z158m Fc DNA

Both groups of four mice generate comparable antibody response profiles with all mice generating much lower levels of antibody when compared to the wild type construct. **Figures 4.21a-c** and **4.22a-c** display the isotyping results obtained for both groups. Comparison of isotype ratios indicates that concentrations are similar indicating a mixed IgG_1/IgG_{2a} profile. These results suggest that incorporation of the Z158m mutation into the SCR 1-3 Fc DNA construct switches the type of response generated and prevents the production of CD55 specific antibodies.

Figure 4.21: Isotyping of antibody responses generated by SCR 1-3 Z158m Fc (Group 1);

Figure 4.21a: Standard curve for CD55 specific isotyped antibodies

Standard curve for mouse anti-CD55 specific antibodies was set up using Fc captured SCR 1-3 Fc and titrated concentrations of CD55 specific antibodies BRIC 216 (IgG_1) and IA10 clone (IgG_{2a}) bound to 96 well ELISA plates.

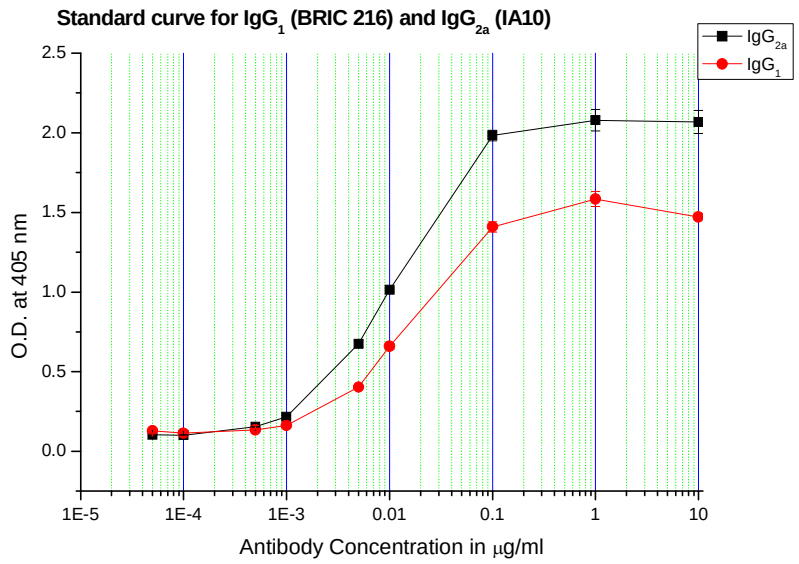
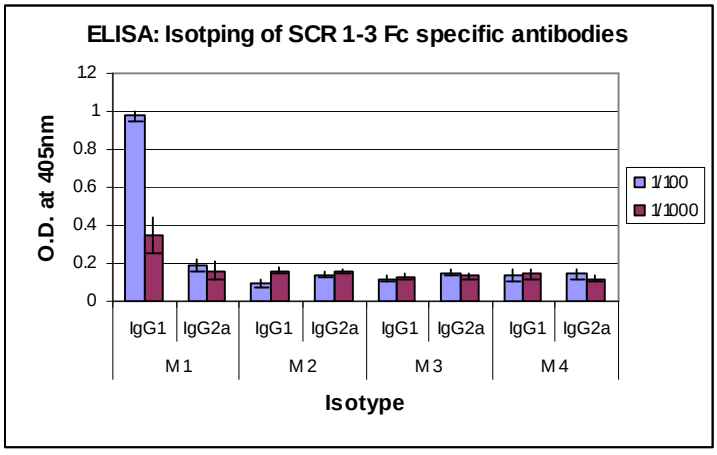


Figure 4.21b: Concentration of isotyped antibody responses



CD55 isotyped responses determined via ELISA assessment of SCR 1-3 Z158m Fc DNA immunised mouse sera. Titrated responses observed against SCR 1-3 Fc protein and utilising horse radish peroxidase conjugated goat anti mouse IgG_1 and IgG_{2a} antibodies with ABTS substrate system, measuring absorbance at 405nm. Standard deviation expressed as range of two data points.

Figure 4.21c: Antibody concentrations extrapolated from standard curve and antibody screen results

	IgG_1		IgG_{2a}	
	1/100	**1/1000**	**1/100**	**1/1000**
M1	0.012µg/ml	0.0014µg/ml	0.0001µg/ml	<0.0001µg/ml
M2	<0.0001µg/ml	<0.0001µg/ml	<0.0001µg/ml	<0.0001µg/ml
M3	<0.0001µgml	<0.0001µg/ml	<0.0001µgml	<0.0001µg/ml
M4	<0.0001µg/ml	<0.0001µg/ml	<0.0001µg/ml	<0.0001µg/ml

The Z158m mutation within the SCR 1-3 Fc DNA vaccine appears to have class switched the nature of response generated. The ratio between antibody isotypes is no longer defined and there is a greater level of IgG_{2a} antibodies present, indicating the presence of a more mixed Th1/2 type response.

Figure 4.22: Isotyping of antibody responses generated by SCR 1-3 Z158m Fc (Group 2)

Figure 4.22a: Standard curve for CD55 specific isotyped antibodies

Standard curve for mouse anti-CD55 specific antibodies was set up using Fc captured SCR 1-3 Fc and titrated concentrations of CD55 specific antibodies BRIC 216 (IgG_1) and IA10 clone (IgG_{2a}) bound to 96 well ELISA plates.

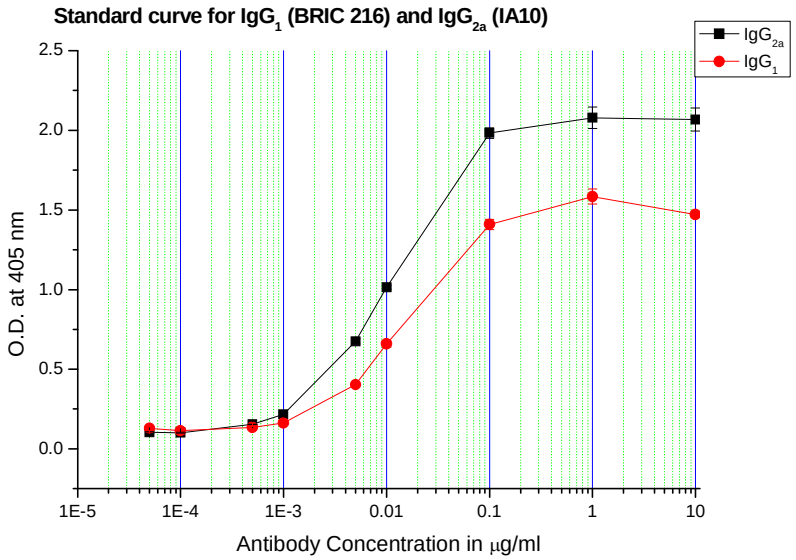
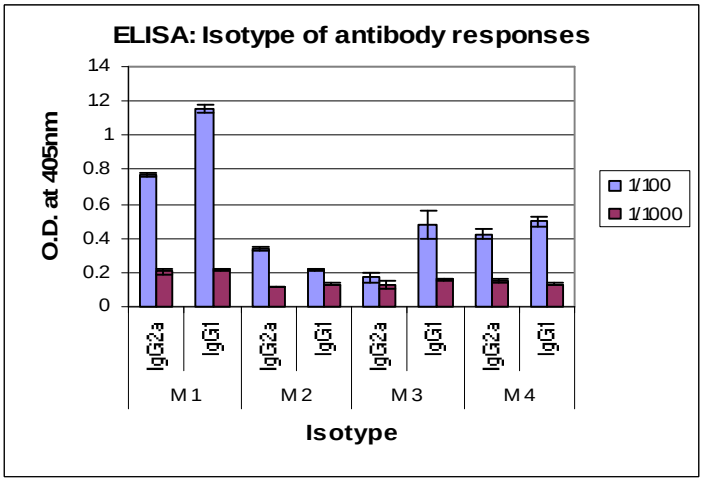


Figure 4.22b: Concentration of isotyped antibody responses



CD55 isotyped responses determined via ELISA assessment of SCR 1-3 Z158m Fc DNA immunised mouse sera. Titrated responses observed against SCR 1-3 Fc protein and utilising horse radish peroxidase conjugated goat anti mouse IgG_1 and IgG_{2a} antibodies with ABTS substrate system, measuring absorbance at 405nm. Standard deviation expressed as range of two data points.

Figure 4.22c: Antibody concentrations extrapolated from standard curve and antibody screen results

	IgG_1		IgG_{2a}	
	1/100	**1/1000**	**1/100**	**1/1000**
M1	0.015µg/ml	0.001µg/ml	0.011µg/ml	0.001µg/ml
M2	0.001µg/ml	0.0001µg/ml	<0.0012µg/ml	~0.0001µg/ml
M3	0.0018µgml	0.0001µg/ml	~0.0001µgml	~0.0001µg/ml
M4	0.0019µg/ml	0.0001µg/ml	0.0015µg/ml	~0.0001µg/ml

The Z158m mutation within the SCR 1-3 Fc DNA vaccine appears to have class switched the nature of response generated. The ratio between antibody isotypes is no longer defined and there is a greater level of IgG_{2a} antibodies present, indicating the presence of a mixed Th1/2 type response.

4.10: Antibody specificity to CD55 natively expressed on tumour targets

In order to assess the potential therapeutic nature of generated antibodies, sera were screened for ability to recognise native CD55 expressed on the human osteosarcoma cell line 791T, in comparison to the CD55 negative colorectal carcinoma line Colo 205. **Figure 4.23a-d** show the results obtained from direct labelling of 1µg of control CD55 specific antibodies compared to 50µl of 1/100 diluted immunised serum

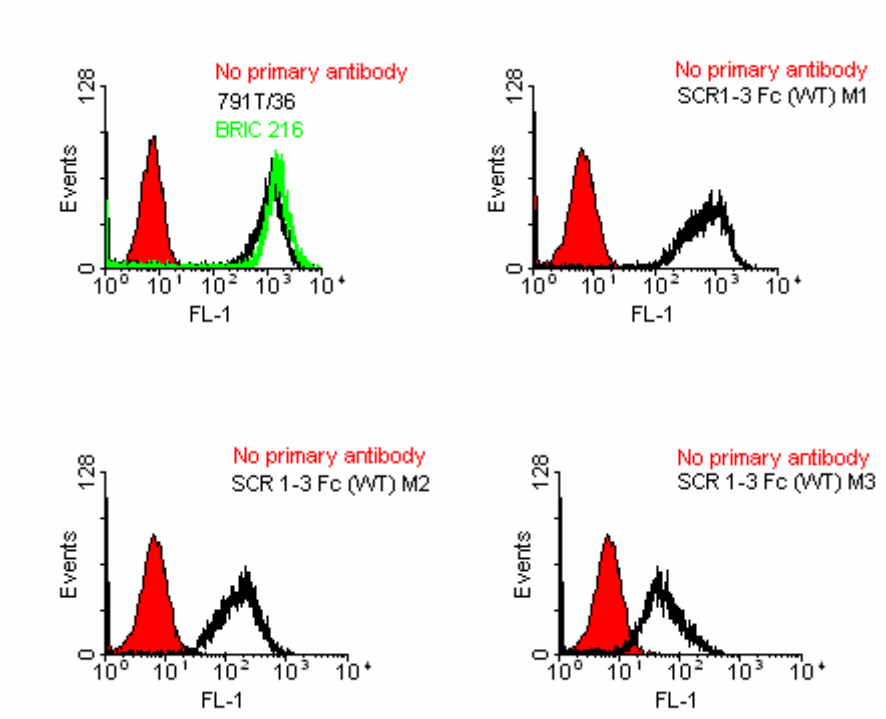
applied to both cell types. Secondary rabbit anti mouse FITC conjugated antibodies were used to enable analysis of binding via flow cytometry. Sera from all four mice of the SCR 1-3 (WT) Fc immunisation group 1 show high levels of binding to CD55, displaying up to 3 $\log^{10}$ greater staining compared to un-immunised sera responses. Results of sera obtained from mouse 2 of group 1 and all four mice of group 2 SCR 1-3 Z158m Fc immunisation protocol indicate no recognition of naturally expressed CD55 with all values comparable with un-immunised sera. No binding of antibodies is observed to the CD55 negative line Colo 205, with results comparable with un-immunised sera and negative controls.

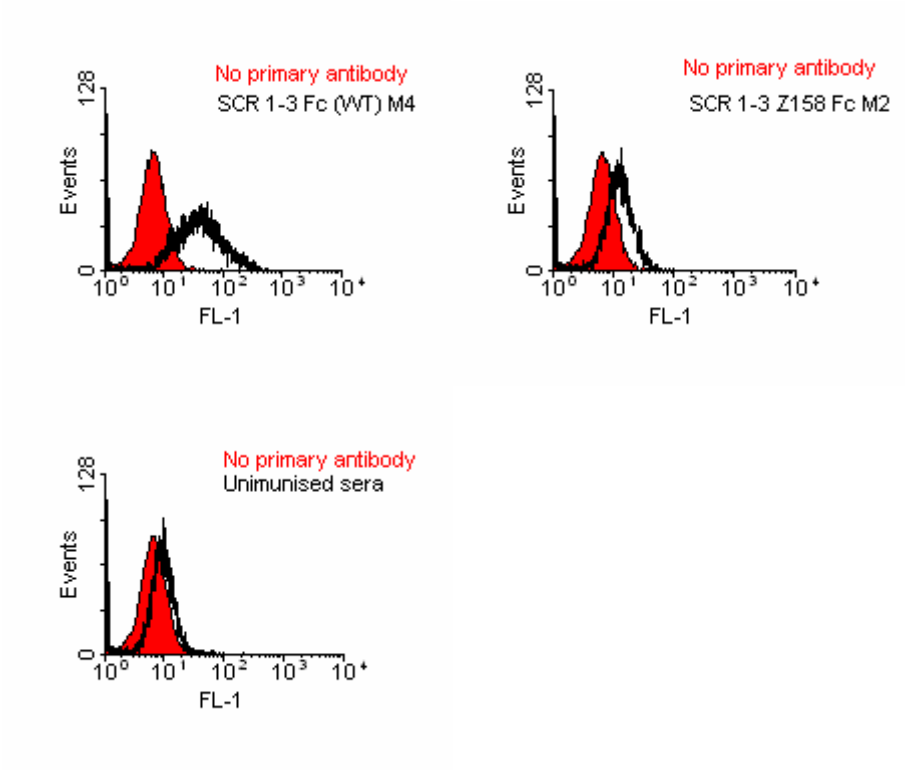
These results indicate that SCR 1-3 (WT) Fc DNA immunisations generate antibody responses with CD55 specificity that recognise natively expressed antigen on a tumour target cell line. In comparison, the mutant SCR 1-3 Z158m Fc DNA immunisations generally fail to drive CD55 specific antibody mediated responses.

Figure 4.23a: FACS Binding Assay of sera from SCR 1-3 Fc (WT) and Z158m DNA immunisations, antibody recognition of natively expressed CD55 on the surface of tumour cell lines.

791T and Colo 205 cells labelled with 1µg of CD55 specific antibodies and 50µl of 1/100 diluted immunised sera. Rabbit anti-mouse FITC conjugated antibodies added as secondary label. Cells analysed by flow cytometry.

791T cells (CD55 positive)





SCR 1-3 Z158m Fc Immunisation (Group 2)

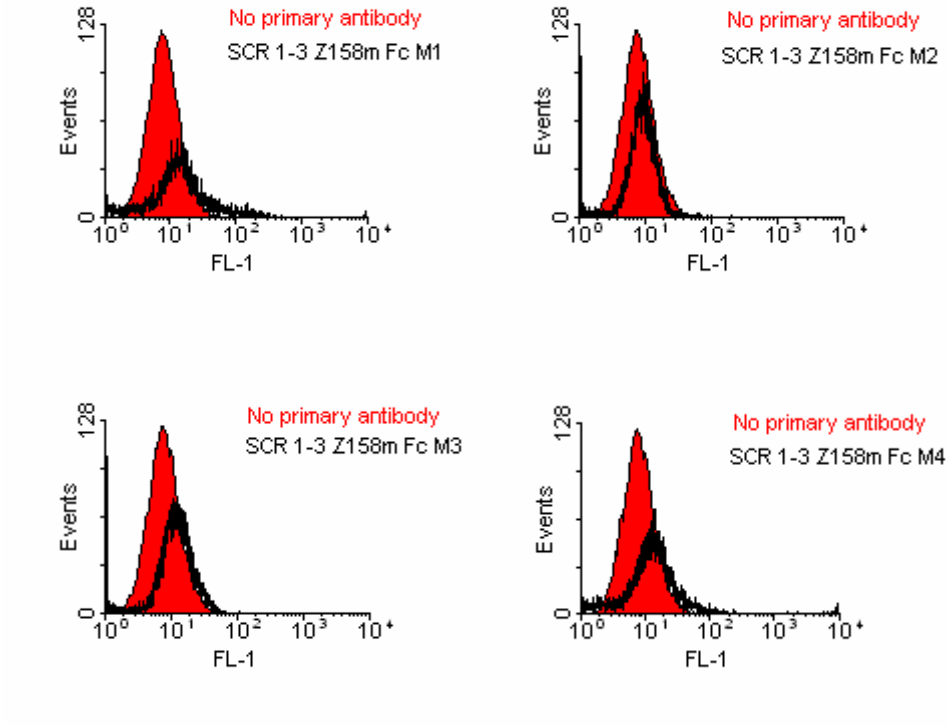
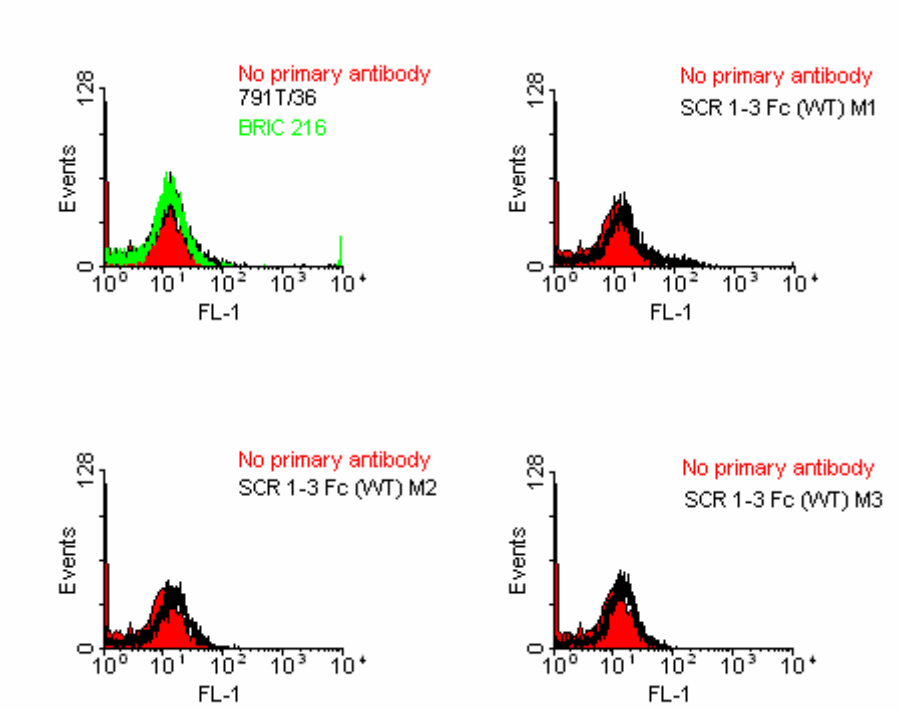


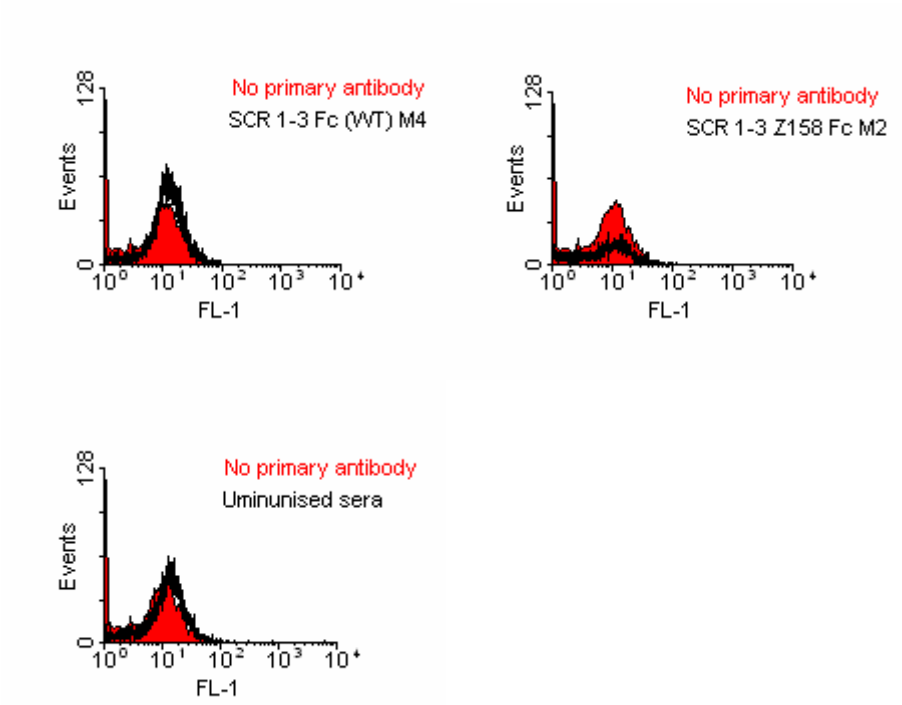
Table 4.23b: Summary of Fluorescence values generated for sera binding assay

	Mean Linear Fluorescence	Geometric Mean
No primary antibody	8.22	5.46
791T/36	1068.37	548.52
BRIC 216	1608.91	799.6
Group 1		
SCR 1-3 Fc (WT) M1	759.63	366.23
SCR 1-3 Fc (WT) M2	166.27	92.63
SCR 1-3 Fc (WT) M3	68.52	38.24
SCR 1-3 Fc (WT) M4	59.29	30.92
SCR 1-3 Z158m Fc M2	14.61	10.17
Unimunised sera	17.36	7.47
Group 2		
SCR 1-3 Z158m Fc M1	13.71	10.32
SCR 1-3 Z158m Fc M2	11.63	10.47
SCR 1-3 Z158m Fc M3	13.24	10.09
SCR 1-3 Z158m Fc M4	13.57	10.23

Binding of BRIC 216 and 791T/36 indicate the level of CD55 expression on 791T cells. Wild type SCR 1-3 Fc immunised sera shows a high degree of binding and specificity to native CD55 as expressed by 791T cells. The Z158m DNA construct appears to generate antibodies of limited specificity to CD55.

Figure 4.23c: Colo 205 cells (CD55 negative)





SCR 1-3 Z158m Fc (Group 2)

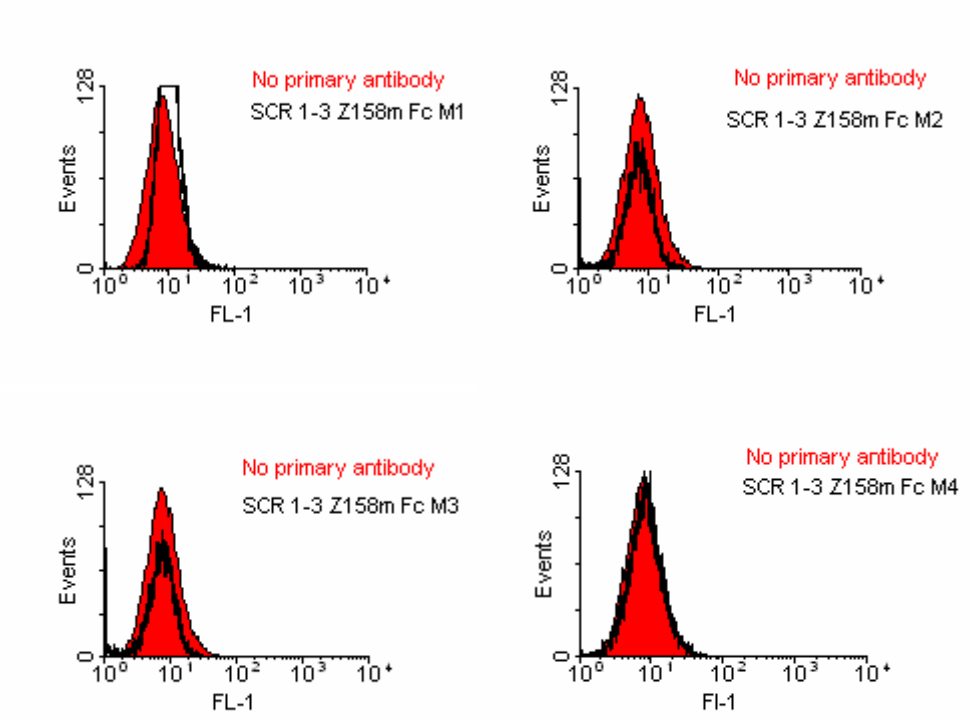


Figure 4.23d: Summary of fluorescence values generated for sera binding assay

	Mean Linear Fluorescence	Geometric Mean
No primary antibody	10.23	6.12
791T/36	82.62	10.57
BRIC 216	94.97	9.68
Group 1		
SCR 1-3 Fc (WT) M1	45.91	11.48
SCR 1-3 Fc (WT) M2	17.38	10.17
SCR 1-3 Fc (WT) M3	17.52	10.51
SCR 1-3 Fc (WT) M4	15.57	9.87
SCR 1-3 Z158 Fc M2	11.54	5.46
Unimunised sera	24.4	9.28
Group 2		
SCR 1-3 Z158m Fc M1	12.12	7.67
SCR 1-3 Z158m Fc M2	11.72	9.43
SCR 1-3 Z158m Fc M3	10.78	10.92
SCR 1-3 Z158m Fc M4	10.04	7.13

As Colo 205 cells do not express CD55 minimal background binding is observed both with control antibodies and immunised sera.

4.11: Discussion

Many successful DNA vaccination trails have shown that significant antibody responses can be elicited towards a vaccinating antigen as reviewed by Hanlon et al., 2001. Both the efficacy and nature of DNA elicited responses can be affected by many factors including the route of delivery (Pertmer et al., 1996), the expression vector and the form of the DNA encoded antigen (secreted, intracellular or membrane associated). However, current opinion suggests that the most important factor influencing raised responses is the nature of the expressed antigen itself. This is shown with early studies identifying that influenza haemaglutinin antigen is capable of generating long lived responses in mice (Robinson et al., 1997) compared with an HIV antigen that raised only transient, low level titres in mice (Lu et al., 1996). In conclusion, the basic differences in physical structure of antigens and how this influences interactions within the immune system itself appear to dictate the nature of specific responses generated (Robinson et al., 1997). T helper responses are critical in driving immune responses, with Th_1 characterised by IFNγ synthesis in conjunction with IL-2 production mediating cellular responses and Th_2 that are characterised by IL-4 production driving humoral immunity. Of direct relevance to the current study is the ability to drive Th_2 responses activating non phagocytic defences, such as mast cells while assisting B cells to produce IgG_1 antibodies. While the class of response generated is associated, in part by the nature of the antigen, arguments exist stating that Th_1 and Th_2 responses are based upon differing cytokine expression patterns (reviewed by Hanlon et al., 2001).

Post DNA vaccination, internally expressed antigens are presented by both MHC classes, eliciting both humoral and cellular responses. In the current context, administration of plasmid DNA via gene gun is capable of driving responses through direct transfection of APCs, which are capable of processing and presenting antigen through both class I and class II pathways, and through transfection of non-APCs such as muscle cells and keratinocytes, which express whole antigen which is subsequently transferred to professional antigen presenting cells.

Harris et al (2002) support the methodology incorporated into this study. They generated fusion proteins, via transfection of CHO cells, consisting of the CD55 complement control regions formed as 'antibody like' molecules containing antigen moieties linked to antibody Fc, in the place of Fab arms. Their experimentation proved that efficient generation of soluble forms of proteins conveniently 'tagged' with Fc domains could be achieved, enabling efficient purification from crude tissue culture supernatant by protein A affinity chromatography. They also show that immunisation with CD55 fusion proteins can generate antibodies specific to the CD55 short consensus repeats incorporated into the immunogen, and successful screening of responses could be obtained utilising the fusion proteins as a viable target. However, in comparison with results obtained in this study, antibodies generated in response to their human CD55 Fc protein also cross reacted with their control fusion protein, indicating reactivity with the common Fc domain. This is in contrast with the current study utilising a SCR 1-3 (WT) Fc DNA construct, eliciting CD55 specific antibody responses. Interestingly, Harris indicates that epitopes located near the hinge region of the fusion protein are masked by steric effects, which prevent antibody generation specific to these domains. In their example, the protein contained all four SCR domains of CD55 and no antibodies were produced specific to the fourth carboxy-terminal SCR. They state that in order to obtain mAbs to such areas of protein that may be masked, the inclusion of spacer regions is important to remove the potential target from the shadow of the Fc. With respect to this project, the development of IgG_1 antibodies, specific to CD55, were desirable for potential induction of ADCC responses to tumour targets bearing CD55. The development of antibodies specific to the SCR 1-2 region, capable of inhibiting the decay accelerating activity of CD55, would also be desirable, and as these domains are separated from the Fc region within our construct by the SCR 3 region, the potential for masking would be reduced.

Analysis of the SCR 1-3 (Wild Type) Fc DNA construct indicates that a CD55 specific Th_2 response is generated, producing IgG_1 antibodies capable of recognising native antigen expressed by target tumour cells. In contrast, the anchor modified construct containing the Z158m mutation appears to generate a mixed Th type response, eliciting both IgG_1 and IgG_{2a} antibodies, possessing general specificity to both the CD55 domain and the human IgG Fc region of the construct. Antibodies generated by this mutant vaccine also fail to recognise natively expressed protein on

the surface of tumour cells. This poses an interesting question of whether the Z158m mutation actually confers conformational changes within the expressed protein, affecting the generation of functional antibodies. The Z158m mutation is located in the SCR 1 region of CD55 and it is suggested that this point mutation potentially alters the complete tertiary structures of both the SCR 1 and 2 domains. In order to confirm this suggestion, antibody clones would need to be isolated along with their specific targets, determining whether the mutation prevents all responses to the CD55 domains. The results obtained also suggest a potential switch in class of response generated when comparing the wild type construct with the mutated variant, although this may not be a true comparison considering that the Z158m vaccine generates mixed Fc/CD55 specificity. While further assessment of these findings need to be carried out, it is clear that the wild type construct generates antibodies with therapeutic potential. Functional blocking studies also need to be carried out in the form of complement deposition assays, in order to assess the potential for inhibition of CD55 decay accelerating activity on tumour targets. This could be achieved utilising c1q depleted serum, confirming whether increased complement deposition was due to ADCC or prevention of CD55 activity. The potential for whole antigen/protein immunisation should also be assessed, when considering other works this high level of antigen administration successfully boosts responses obtained (Harris et al., 2002). A final assessment of the therapeutic potential of the DNA vaccine, and the antibodies produced, would be via a lytic assay determining if direct cell killing could be mediated by the antibody populations produced.

Chapter 5: Development of an SCR 1-3 Fc DNA vaccine incorporating heteroclitic epitopes in order to stimulate CTL mediated responses

5.1: Introduction

DNA immunisation strategies have been shown to generate efficient immune responses based on the fact that foreign protein is expressed directly in or is quickly taken up by professional antigen presenting cells (Takashima and Morita, 1999). Also, relatively small numbers of activated APCs have been shown following immunisation to be sufficient at stimulating T cell responses (Casares et al., 1997).

In order to develop a successful anti tumour vaccine, exploitation of potential low affinity T cells which have escaped thymic tolerance into the periphery, needs to be achieved. Identification of potential CTL epitopes from within tumour-associated antigens, such as CD55, would be an effective method of driving immune responses. Many such epitopes are shown to bind to MHC with only moderate affinity and therefore initiate poor antigen specific responses. Several studies have shown that by modifying anchor residues to preferred amino acids that MHC/peptide interactions can be significantly increased without altering the T cell epitope and improve the frequency and avidity of immune responses (Berzofsky et al., 2001).

It has been demonstrated that uptake of antibody/antigen complexes via the CD64 receptor results in 1,000 fold enhancement of the presentation of peptides on MHC class II (Cella et al., 1997). Internalisation of this receptor has also been shown to activate dendritic cells resulting in increased expression of co-stimulatory molecules and cross priming of antigens on class I MHC (Regnault et al., 1999). Therefore, targeting of antigens to this receptor could improve expression of epitopes upon the MHC of activated dendritic cells whilst providing both signals 1 and 2 in order to activate moderate avidity T cells. As previous studies highlight the success of Fc targeting of dendritic cells, the SCR 1-3 domain of human CD55 was cloned in sequence with human Fcγ1.

Heteroclitic epitopes were identified from within human CD55 and incorporated into a DNA vaccine in sequence with a human IgG Fc tail in order to assess the responses generated in HLA-A*201 HHD II transgenic mice.

Results

5.2: Identification of potential CTL epitopes for vaccine development

Several Internet based epitope prediction algorithms were utilised to identify potential moderate to high affinity HLA_A*201 epitopes from within the protein sequence of human CD55. Five programs (**section 2.1.2**) were utilised in order to generate a consistent range of sequences predicted not only to effectively bind MHC but also to show a high probability of native expression. **Tables 5.1a-e** indicate the top 14 highest ranking predicted epitopes displaying epitope start positions, relative to the N terminal end of the sequence, and the relative score as allocated by each database. The 14 highest ranked sequences are shown, as epitopes generating lower scores were determined to be of too low affinity for potential exploitation.

Analysis of the results generated identified clear trends in the predicted binding epitopes. The amino acid leucine occurs at a disproportionately high frequency when compared with other residues, occupying 31% of all residues identified by the MHCPEP program. This supports the basis on which the databases are generated suggesting that high leucine content confers enhanced binding properties upon a given peptide sequence. However, this observation may also reflect the relatively high frequency of this residue within the CD55 protein sequence. Leucine and valine have also been previously identified as favourable residues at positions 2 and 9 of peptide sequences conveying elevated class I binding affinities (Sette et. al., 1994). The results generated support these findings with many of the top ranking epitope sequences containing one or both of these residues in the corresponding loci.

Figure 5.1: HLA-A*201 epitope prediction

Several internet based epitope prediction algorithms were utilised to identify potential moderate to high affinity HLA class I epitopes from within the CD55 protein sequence. All the sequence databases used employ arbitrary units, ranking peptide sequences in order of predicted affinity relative to reference peptides, and as such, generated results can not be directly compared across databases. Analysis was carried out incorporating the following parameters: Peptides containing 9 amino acids assessing affinity within the HLA-A*201 haplotype. In all tables, “Position” equates to the location of the first amino acid in the epitope sequence in reference to the N-terminal end of the primary protein sequence of CD55. See **section 2.1.2** for references and website addresses. The 14 highest ranking epitopes are shown for all program results as epitopes below this ranking were determined to be of too low

affinity for potential exploitation. Highlighted epitopes correlate with sequences identified for further analysis.

Table 5.1a: Results obtained from the MHCPEP program

MHCPEP				SYFPEITHI		
Rank	**Epitope**	**Position**	**Score**	**Epitope**	**Position**	**Score**
1	ELPRLLLLV	16	0.83	ELPRLLLLV	16	0.82
2	LLGELPRLL	13	0.75	PLLGELPRL	12	0.78
3	LLLLVLLCL	20	0.68	LLLLVLLCL	20	0.78
4	PLLGELPRL	12	0.51	SLSPKLTCL	137	0.66
5	RLLLLVLL	19	0.49	LLGELPRLL	13	0.55
6	KLFGSTSSF	194	0.46	RLLLLVLLC	19	0.38
7	GLPPDVPNA	36	0.32	LVLLCLPAV	23	0.24
8	SLSPKLTCL	137	0.26	SLKQPYITQ	107	0.19
9	ALEGRTSFP	47	0.22	ILFGATISF	179	0.15
10	ILFGATISF	179	0.16	YITQNYFPV	112	0.08
11	YITQNYFPV	112	0.1	GELPRLLLL	15	0.07
12	LLVLLCLPA	22	0.06	KIPGEKDSV	70	0.05
13	VLLCLPAVW	24	0.06	KLFGSTSSF	194	0.04
14	FCLISGSSV	202	0.05	GLPPDVPNA	36	0.02

This program incorporates two databases while allocating scores in equivalent units enabling direct comparison of obtained results.

Table 5.1b: Results obtained from the SYFPEITHI program

Rank	Epitope	Position	Score
1	LLLLVLLCL	20	29
2	SLSPKLTCL	137	29
3	AALPLLGEL	9	27
4	PLLGELPRL	12	26
5	LLGELPRLL	13	25
6	LVLLCLPAV	23	24
7	ELPRLLLLV	16	23
8	KIPGEKDSV	70	23
9	GLPPDVPNA	36	22
10	SVPAALPLL	6	21
11	GELPRLLLL	15	20
12	LLLVLLCLP	21	19
13	YITQNYFPV	112	19
14	QIDVPGGIL	172	19

The SYFPEITHI program is widely used and referenced. This database is predominantly utilised within our laboratory as it often produces consistent results with an acceptable degree of accuracy.

Table 5.1c: Results obtained from the HLA ligand/motif database

Rank	Epitope	Position	Score
1	LLLLVLLCL	20	161
2	ELPRLLLLV	16	159
3	PLLGELPRL	12	152
4	LLGELPRLL	13	147
5	SLSPKLTCL	137	142

6	RLLLLVLLC	19	130
7	LLLVLLCLP	21	122
8	LLVLLCLPA	22	119
9	ALPLLGELP	10	114
10	GLPPDVPNA	36	114
11	ILFGATISF	179	111
12	LVLLCLPAV	23	106
13	KLFGSTSSF	194	106
14	GELPRLLLL	15	108

Table 5.1d: Results obtained from the BIMAS program

Rank	Epitope	Position	Score
1	YITQNYFPV	112	382.73
2	LLLLVLLCL	20	309.73
3	LVLLCLPAV	23	88.04
4	CLQNLKWST	144	55.89
5	SLSPKLTCL	137	49.13
6	RLLLLVLLC	19	42.28
7	LLGELPRLL	13	29.78
8	KIPGEKDSV	70	16.40
9	ELPRLLLLV	16	11.99
10	GLPPDVPNA	36	11.43
11	LLVLLCLPA	22	8.45
12	FCLISGSSV	202	7.73
13	SVPAALPLL	6	4.23
14	PLLGELPRL	12	3.99

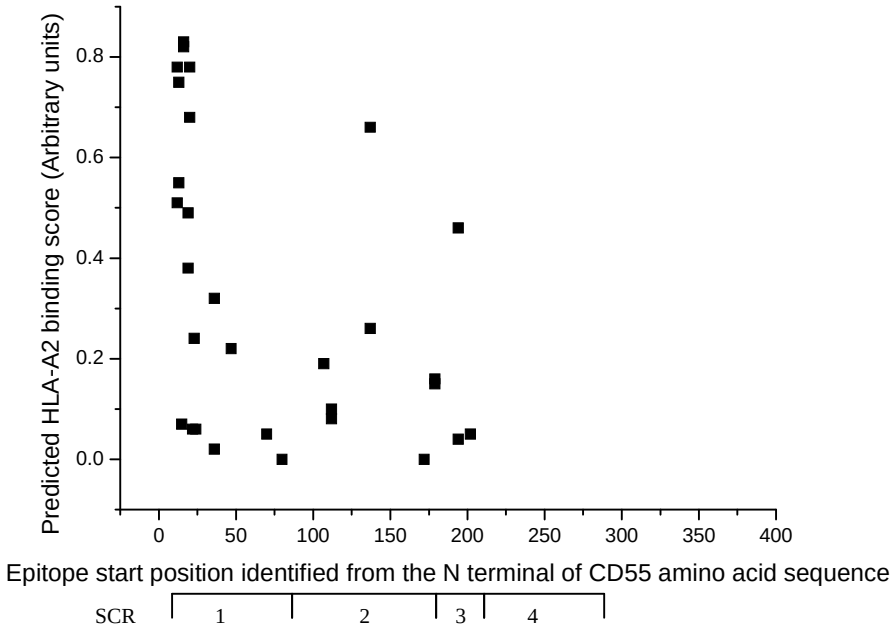
Table 5.1e: Results obtained from Bioinformatics at Leeds (MHC prediction) database

Rank	Epitope	Position	TAP Score	MHC Score	Overall Score
1	LLLLVLLCL	20	5.1	111.2	21.8
2	RSCEVPTRL	95	5.6	111.8	20.1
3	SVPAALPLL	6	6.5	111.9	17.3
4	TYKCEESFV	61	6.8	112.8	16.7
5	LTCLQNLKW	142	6.4	106.9	16.7
6	VLLCLPAVW	24	7	115.2	16.6
7	SLSPKLTCL	137	6.8	107.7	15.7
8	VICLKGSQW	78	7	108.2	15.5
9	GEKDSVICL	73	7	106.6	15.2
10	REPSLSPKL	134	8.1	119	14.8
11	ILFGATISF	179	7.5	108.6	14.6
12	SASLKQPYI	105	7.8	113.1	14.5
13	SFPEDTVIT	53	7.6	108.9	14.4
14	QWSDPLPEC	211	7.6	108.5	14.3

This program generates TAP scores in conjunction with relative predicted binding affinity. TAP values represent the probability that each peptide will be generated from the native CD55 protein sequence by the proteosome complex. Lower scores reflect a greater probability of peptide production. The overall score is obtained by dividing the MHC score by the TAP value, therefore high scores are indicative of elevated probability that the identified sequence will be processed and presented by HLA-A*201^{+} cells that encounter CD55 protein in it's native state.

Figure 5.2 is a scatter plot of the top 28 ranked epitopes identified by the MHCPEP program and correlates their relative start position in the CD55 sequence with their predicted HLA-A2 binding score. The locations of predicted epitopes within CD55 appear to predominantly occur towards the N terminal of the complete protein sequence. Potential preferred epitopes are exclusively identified in the Leader-SCR 1-3 domains of CD55, with the greatest frequency found across the leader-SCR 1 region.

Figure 5.2: Location of CD55 HLA-A2 epitopes as identified by MHCPEP program (top 28)



Epitopes identified from the MHCPEP program were correlated with their relative start positions within the CD55 protein sequence. The top 28 identified epitopes were analysed in order to assess which domains, if any, contained the most moderate to high affinity epitopes.

The various programs generate a variety of predicted results, as could be expected with their independent rule and scoring systems. This can clearly be discerned by comparison of the epitope scores produced by the two SYFPEITHI algorithms, with some sequences gaining greater binding and ranking scores and other epitopes only being identified by one program. Upon further analysis, trends in predicted binding affinities of certain epitopes can be identified with comparative scores and ranked positions. **Table 5.3** shows a summary of the top ten identified epitopes which were consistently identified from the various prediction programs. The table shows the amino acid sequence identified and its relative ranked position from each program.

Results from the Bioinformatics at Leeds database are not included in the summary as most of the peptides generated are not identified by the other programs, with the exception of SLSPKLTCL, LLLLVLLCL and an overlap of the region VLLCLPAVW, all of which were identified as predicted high affinity HLA binders by alternate systems and were identified for potential modification.

Table 5.3: Summary of top ten ranked epitopes

Epitope	Position	MHCPEP		HLA	BIMAS	SYFPEITHI
		SYFPEITHI	MHCPEP	L/M		
ELPRLLLLV	16	1	1	2	9	7
PLLGELPRL	12	2	4	3	14	4
LLLLVLLCL	20	3	3	1	2	12
SLSPKLTCL	137	4	8	5	5	2
LLGELPRLL	13	5	2	4	7	5
RLLLLVLLC	19	6	5	6	6	----
ILFGATISF	179	9	10	11	----	----
YITQNYFPV	12	10	11		1	13
LVLLCLPAV	23	7	12	12	3	6
GLPPDVPNA	36	14	7	10	10	9

Table displays the top ten wild-type epitopes from within the CD55 protein sequence which were identified as sources for anchor modification and potential vaccine development. Scores shown indicate the rank identified by the relative database.

Due to the apparent consistency of predictions by the MHCPEP algorithms, identifying many of the highest ranked epitopes from all other programs utilised, this database was used in all future assessment of epitopes evaluated. The SYFPEITHI website was also used for future comparisons of modified sequences. BLAST (NIH) assessment was carried out on identified sequences in order to confirm that they were unique to the CD55 protein sequence.

5.3: Generation and assessment of heteroclitic epitopes for HLA-A201

Having identified potential heteroclitic epitopes from within the protein sequence of CD55, all of which displayed predicted moderate to high affinity MHC binding potential, modification of anchor residues was desired to increase peptide affinity for the MHC molecule in order to enhance immunogenicity. Mutation of anchor residues on HLA-A2 binding epitopes to known preferred amino acids has been shown to increase the affinity for MHC without altering the T cell epitope (Ahlers et al 2001, Irvine et al 1999, Sarobe et al 1998), and thus jeopardising the affinity/avidity of the T cell- MHC peptide interaction.

Therefore, binding predictions were ascertained for point mutations within the epitope sequence for tyrosine, leucine and valine at positions 1, 2 and 9 respectively. The potential for multiple mutations was also assessed with results displayed in **figure 5.4**.

Figure 5.4: Predicted binding scores of anchor modified CD55 derived peptides

Wild-type	Anchor modified sequence	MHCPEP		SYFPEITHI Score
		SYFPEITHI score	MHCPEP score	
ELPRLLLLV		0.82	0.83	23
	YLPLLLLV	1.08	1.04	28
PLLGELPRL		0.51	0.78	26
	PLLGELPR**V**	0.99	0.88	26
	YPPGELPRL	0.77	0.83	31
	YLLGELPR**V**	1.1	1.06	31
LLLLVLLCL		0.78	0.68	29
	LLLLVLLC**V**	1.09	0.82	29
	YLLLVLLCL	0.91	0.86	29
	YLLLVLLC**V**	1.15	1.05	29
SLSPKLTCL		0.66	0.26	29
	SLSPKLTC**V**	0.78	0.5	29
	YLSPKLTCL	0.63	0.6	29
	YLSPKLTC**V**	0.74	0.8	29
LLGELPRLL		0.55	0.75	25
	LLGELPRL**V**	0.83	0.92	25
	YLGELPRLL	0.68	0.86	25
	YLGELPRL**V**	0.91	1.08	25
RLLLLVLLC		0.77	1.07	18
	RLLLLVLL**V**	0.99	1.2	28
	YLLLLVLLC	0.47	0.56	20
	YLLLLVLL**V**	1.1	1.23	30
ILFGATISF		0.15	0.16	19
	ILFGATIS**V**	0.98	0.88	29
	YLFGATISF	0.233	0.44	19
	YLFGATIS**V**	1.03	1.15	29
YITQNYFPV		0.08	0.1	19
	Y**L**TQNYFPV	0.51	0.77	21
LVLLCLPAV		0.24	0.06	24
	YVLLCLPAV	0.31	0.1	24
	L**L**LLCLPAV	0.97	0.63	30
	YLLLCLPAV	0.89	0.62	30
GLPPDVPNA		0.02	0.32	22
	YLPPDVPNA	0.46	0.52	23
	GLPPDVPN**V**	0.61	0.87	28
	YLPPDVPN**V**	0.72	0.76	29

The MHCPEP and SYFPEITHI databases were utilised to generate predicted binding scores for all anchor modified epitopes. Primary anchor and P1Y variants were analysed in conjunction with compound mutated epitopes. Point mutations are identified in bold.

The results displayed indicate the ten identified wild type epitopes along with their respective anchor modification and predicted binding scores as allocated by the SYFPEITHI and MHCPEP programs. The MHCPEP system appears to generally predict greater increase in affinity scores when compared to the SYFPEITHI database for all mutated peptides. Five of the wild type epitopes appear to be significantly enhanced by anchor modification when relative predictions are assessed: ELPRLLLLV, PLLGELPRL, ILFGATISF, YITQNYFPV and LVLLCLPAV all show increased affinity scores with all chosen mutations. Interestingly, many of the primary sequences already incorporated leucine at position 2, being identified as a contributing factor to the initial predicted score. Tyrosine however, generally appears to only slightly improve predicted scores when incorporated as a single mutation although in combination with valine at position 9, overall scores are significantly elevated. Valine as single mutation appears to confer the greatest improvement in predicted binding affinity for all wild type epitopes.

Software based predicted binding, while a potential source for identifying successful epitopes can only be utilised as a guideline and *in vitro* assessment of chosen peptides was required. Peptides were synthesised for all 36 epitopes as per **section 2.1.1** and utilised in a T2 $Tap^{-/-}$ stabilisation assay as per **section 2.1.8**. Binding affinity was evaluated based on ability to stabilise the class I MHC molecules of HLA-$A2^{+}$ $Tap^{-/-}$ human T2 lymphoblastoid cells by the addition of varying concentrations of synthesised peptides (100µg and 25µg/ml). Mean stabilisation scores were calculated as a ratio of mean linear fluorescence with peptide divided by M.L.F. without peptide, with results corrected for the presence of DMSO. The scores generated can be evaluated as a fold increase in the level of stabilised MHC molecules present on the cell surface compared to the level present in the absence of stabilising peptides. Thus a value of 1 would indicate no increase in MHC presentation and stabilisation. **Figure 5.5** displays the results obtained for T2 stabilisation assays including repeat analysis of peptides displaying significant increases in MHC binding. To be potentially immunogenic, both wild type and anchor modified peptides need to exhibit high T2 scores with modified peptides potentially eliciting elevated scores in comparison to their wild type counterparts. While there is no proven 'threshold' value above which peptides can be guaranteed to elicit immune responses, T2 values in excess of 2, ideally greater than 3, for both mutant and wild type can indicate immunogenic potential. Several wild type peptides generate scores, which would

identify them for possible vaccine constructs, namely Z142, Z68 and Z158. At a peptide concentration of 100µg/ml, anchor-modified mutants of Z142, Z68 and Z158 all show an increased stabilising affect. Z158 also generated the highest T2 stabilisation value of all wild type epitopes of 4.36, with its Z158m mutant generating a score of 5.29 at 100µg/ml. Interestingly, all three of these mentioned peptides generate high stabilising scores at the lower concentration of 25µg/ml with Z158m producing a value of 5.17, again the highest score of all anchor modified peptides.

Figure 5.5: T2 ($Tap^{-/-}$) stabilisation assay

1 x 10^5 T2 cells were incubated for 16 hours at 37°C, 5%CO_2 with specific peptide (100µg/ml and 25µg/ml) in complete medium containing human β_2-microglobulin (100ng/ml). The cells were then incubated for an hour with brefeldin A (0.5µg/ml) and labelled with BB7.2 antibody (HLA-A2 specific). Cells were then labelled with a FITC conjugated goat anti-mouse antibody prior to assessment by flow cytometry. Control wells were set up without peptide and with two concentrations of DMSO (contained in peptide) to enable compensation of results. Histogram plots were obtained and mean linear fluoresce values collated for each sample. Stabilisation scores are calculated as a ratio of M.L.F with peptide/M.L.F without peptide, values corrected for presence of DMSO. Values produced equate to MHC expression as a factor of a base line score of 1. Therefore, results greater than 1 indicate a "fold" increase in MHC molecules present on the surface of labelled cells. Results were obtained in two rounds of experiments, each group consisting of six replicates and 15,000 cells being counted per sample and data is displayed as the mean result obtained. Peptides generating the greatest degree of stabilisation were re-assessed under identical conditions.

Wild-type	Anchor modified sequence	Peptide concentration µg/ml	Stabilisation score (1)	Stabilisation score (2)
ELPRLLLLV		100	1.27	
		25	1.01	
	YLPLLLLV	100	1.23	
		25	1.01	
PLLGELPRL		100	1.34	
		25	1.29	
	PLLGELPR**V**	100	1.46	
		25	1.47	
	YPPGELPRL	100	1.28	
		25	1.21	
	YLLGELPR**V**	100	1.38	
		25	1.27	
LLLLVLLCL		100	1.16	
		25	1.07	
	LLLLVLLC**V**	100	1.19	
		25	1.04	
	YLLLVLLCL	100	1.02	
		25	1.03	
	YLLLVLLC**V**	100	1.07	
		25	1.05	

SLSPKLTCL		100	3.89	3.83
(Z142)		25	3.2	3.15
(Z143)	SLSPKLTC**V**	100	3.92	3.70
		25	3.71	3.89
	YLSPKLTCL	100	2.71	
		25	2.56	
	YLSPKLTC**V**	100	3.65	
		25	3.34	
LLGELPRLL		100	1.34	
		25	1.23	
	LLGELPRL**V**	100	1.64	
		25	1.63	
	YLGELPRLL	100	1.26	
		25	1.07	
	YLGELPRL**V**	100	1.42	
		25	1.37	
RLLLLVLLC		100	1.19	
		25	0.96	
	RLLLLVLL**V**	100	2.69	
		25	1.36	
	YLLLLVLLC	100	1.49	
		25	0.97	
	YLLLLVLL**V**	100	2.21	
		25	1.04	
ILFGATISF		100	4.03	
		25	3.11	
	ILFGATIS**V**	100	1.65	
		25	1.13	
	YLFGATISF	100	1.43	
		25	1.34	
	YLFGATIS**V**	100	1.46	
		25	1.52	
YITQNYFPV		100	1.28	
		25	1.05	
	Y**L**TQNYFPV	100	1.79	
		25	1.63	
LVLLCLPAV		100	2.44	2.55
(Z68)		25	2.08	2.18
	YVLLCLPAV	100	2.37	
		25	2.14	
(Z69)	L**L**LLCLPAV	100	3.03	3.22
		25	2.36	2.23
	YLLLCLPAV	100	2.65	
		25	2.43	
GLPPDVPNA		100	4.36	4.32
(Z158)		25	4.27	3.85
	YLPPDVPNA	100	2.56	
		25	2.43	
(Z158m)	GLPPDVPN**V**	100	5.29	5.17
		25	4.76	4.86
	YLPPDVPN**V**	100	3.32	
		25	2.98	

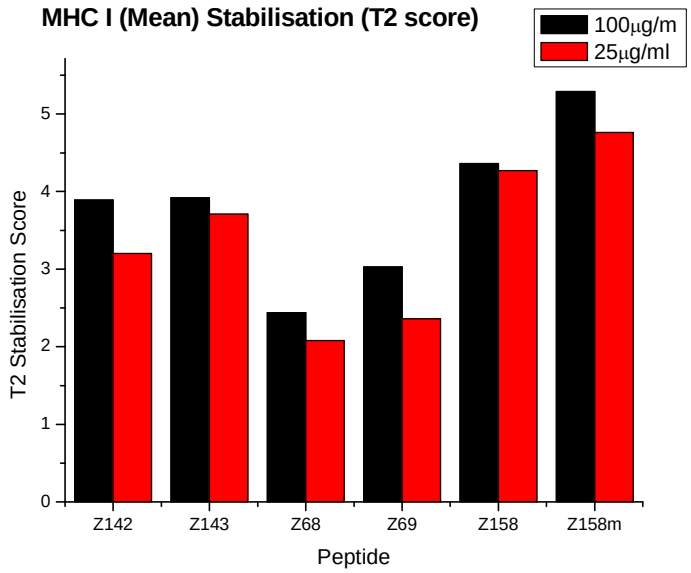
Upon further analysis, several trends appear when considering certain anchor modifications. The Z158m mutant generates a significant increase in MHC stabilisation due to the replacement of an alanine at the P9 position with the much favoured valine residue (Falk et al 1991). Many of the anchor modifications involved the replacement of leucine with valine residues, which are similar in their aromatic structure and potential binding properties, which would possibly indicate why many of the peptides assessed did not generate increased responses. However, this is also the case for both the Z68 to Z69 and Z142 to Z143 mutations, although as in the case for these four peptides the wild type structures did confer significant levels of MHC stabilisation when compared to many of the other wild type peptides screened. This current assessment of anchor modification does appear to support current evidence with six of the wild type peptides modified with valine residues producing elevated, if only slightly, levels of MHC stabilisation. Analysis of P1Y (tyrosine) substitution is also unclear with most of these variants producing T2 stabilisation scores less than their wild type counterparts, with only a few examples of potential improved affinity which may be conferred by the substituted valine residue in compound mutants. **Figure 5.6** displays a summary of the three most successful anchor modifications and their relative predicted and actual stabilisation scores. This data is also graphically represented in **Figure 5.7**, which clearly shows the elevated levels of MHC stabilisation for all three identified peptides with their respective mutants.

Figure 5.6: Summary of peptides generating the greatest level of MHC stabilisation

Epitopes were given identifying codes which were relative to peptides/epitopes previously identified and synthesised within the laboratory.

Epitope	Domain	Sequence	MHCPEP	SYFPEITHI	T2 score 100µg/ml	T2 score 25µg/ml
Z142	SCR2	SLSPKLTCL	0.66	29	3.89	3.2
Z143	SCR2	SLSPKLTC**V**	0.78	29	3.92	3.71
Z68	Leader	LVLLCLPAV	0.24	24	2.44	2.08
Z69	Leader	L**L**LLCLPAV	0.07	30	3.03	2.36
Z158	SCR1	GLPPDVPNA	0.02	22	4.36	4.27
Z158m	SCR1	GLPPDVPN**V**	0.61	28	5.29	4.76

Figure 5.7: Tap$^{-/-}$ stabilisation results for identified wild-type and mutated peptides (six highest scoring)



The T2 stabilisation scores, while allowing a general assessment of each peptide with their various anchor modifications, do not fully display the expression of MHC upon stabilisation. This is due to the mean level of labelled MHC molecules being utilised for the calculation, which does not allow for a range of expression and the general shift being expressed. **Figure 5.8** shows histogram plots for the three most enhanced peptides obtained from the raw data analysed via the fluorocytometric process. These plots clearly show how the addition of peptides to the T2 cells significantly increase fluorescence generated by FITC conjugated anti-HLA-A2 antibodies, representing increased MHC stabilisation.

It is clear from the actual binding observed by both wild type and subsequent modified peptides in comparison to the predicted affinity scores that the software utilised is by no means a definite method of prediction. **Figures 5.9a** and **b** are scatter plots comparing predicted binding scores generated by both the SYFPEITHI and MHCPEP programs with actual binding scores generated from *in vitro* assessment. Both plots indicate that there is little to no correlation between the software generated results and the observed findings. However, analysis of the SYFPEITHI results does show a potential trend of elevated actual binding correlating with increased predicted scores. All peptides identified by the programs do appear to show some level of

binding and thus stabilisation, although it can be seen that peptides predicted to show high levels of binding affinity often produce limited response upon assessment.

Figure 5.8: Histogram plots obtained from FACS data of stabilisation assay results analysed with WINMDI software.

Raw data collected from FACS analysis of labelled T2 cells and the number of events (cells) was plotted against relative FL-1 values. The graphs show results for the three peptides generating the most significant increase in MHC stabilisation. Peptides added at a concentration of 25µg/ml.

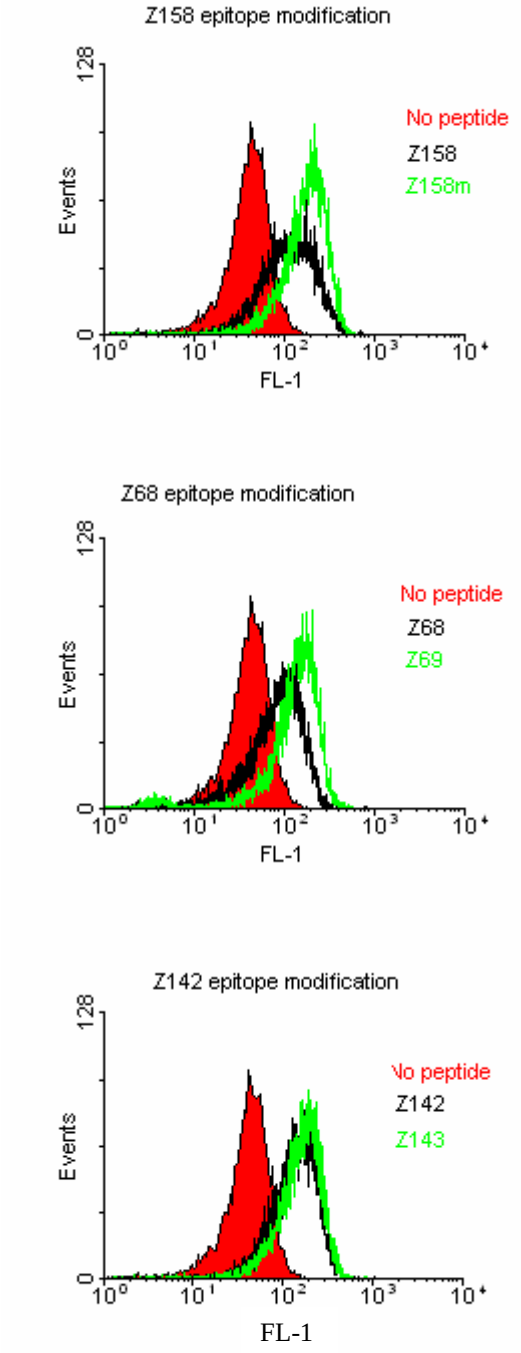


Figure 5.9: Scatter plots of CD55 derived HLA-A2 epitopes predicted binding scores compared with *In Vitro* T2 MHC stabilisation assay results

Predicted binding scores, generated from MHCPEP and SYFPEITHI programs, of wild-type epitopes compared against stabilisation scores obtained from assays of T2 cells incubated with 100µg/ml of target peptides.

Figure 5.9a: Analysis of MHCPEP prediction

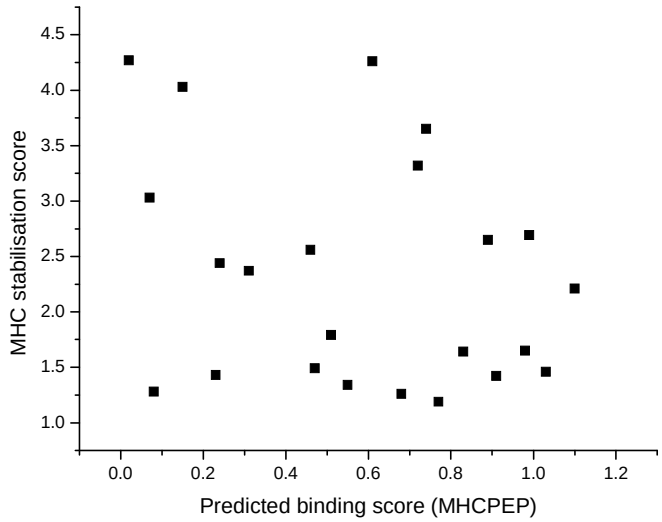
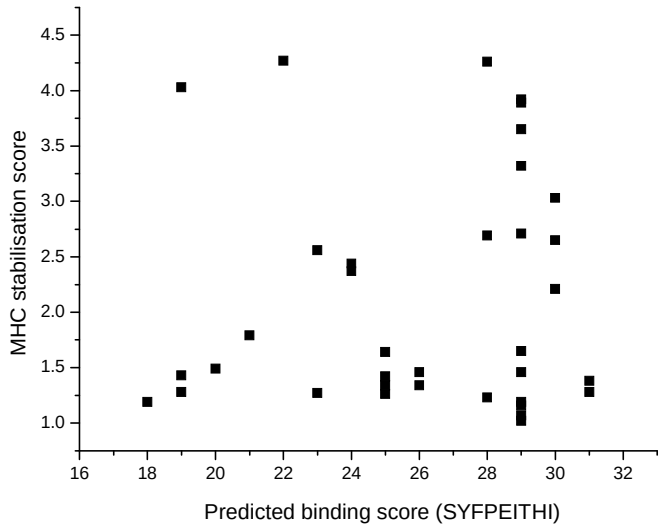


Figure 5.9b: Analysis of SYFPEITHI prediction



5.4: Development of CD55 specific DNA vaccines incorporating heteroclitic epitopes

Having identified potential anchor modified epitopes that successfully bind HLA-A2 MHC molecules, the Z143, Z69 and Z158m sequences were chosen to be incorporated into the existing SCR 1-3 Fc Signal pIgplus DNA construct (see

Chapter 4). Bioinformatics software available at www.ca.expasy.org was utilised to generate and assess primers which would incorporate the desired point mutations into the existing construct. **Figure 5.10** displays how the desired modifications were produced and the sequence of the designed primers, identifying the incorporated mutation and the relative melting temperatures. As Z142 occurred in the SCR 2 domain of CD55 and both Z68 and Z158 both occur within the SCR 1 region, several constructs were developed incorporating combinations of the identified epitopes. Site directed mutagenesis was carried out (**section 2.6.19**) and DNA products were sequenced as per **section 2.6.15** utilising the SCR 3 reverse primer. Large scale production of endotoxin free DNA constructs was carried out on successful mutants as per **section 2.6.14** in preparation for immunisation.

Figure 5.10: Incorporation of modified epitopes into CD55 DNA constructs

Z143, Z69 and Z158m epitopes were selected for incorporation into the SCR 1-3 Fc DNA construct for assessment as a potential vaccine eliciting anti-CD55 immune responses. The sequences were assessed using a BLAST search (NIH, Bethesda, MD) in order to confirm that they were unique to the CD55 protein structure.

Primer design and site directed mutagenesis

Forward and reverse primers were designed to incorporate the desired point mutations into the previously generated SCR 1-3 Fc Signal pIgplus DNA construct. Bioinformatics software available at www.ca.expasy.org was utilised to assess complementary primers and to prevent the possibility of self hybridisation, dimer formation and presence of alternate mutation/binding sites. Site directed mutagenesis was carried out as per **section 2.6.19** upon the SCR 1-3 Fc template DNA and products were sequenced as per **section 2.6.15** utilising the SCR 3 Reverse primer as per **Figure 2.9**. Returned sequences were analysed by NCBI alignment with CD55 protein sequence (accession number BC001288) in order to confirm the presence of desired mutations and to ensure no other mutations within the sequence had occurred. Large scale production of successful mutants was carried out as per **section 2.6.14** generating endotoxin free constructs suitable for DNA immunisation.

Table 5.10a: Primers designed for incorporation of mutated epitopes

CD55 Z143 Forward Primer 5'CA CCA AAA CTA ACT TGC **G**TT CAG AAT TTA AAA TGG TCC ACA GC3' Pro Lys Leu Thr Cys **Val** Gln Asn Leu Lys Trp Ser Thr P K L T C **V** Q N L K W S T Binds 498-540bp of CD55 DNA sequence	**Leu→Val** **L V** 43mer TM=79.21°C
CD55 Z143 Reverse Primer 5'GC TGT GGA CCA TTT TAA ATT CTG AA**C** GCA AGT TAG TTT TGG TG3' Binds 498-540bp of CD55 DNA sequence	 43mer TM=79.21°C
CD55 Z69 Forward Primer 5'CGG CTG CTG CTG CTG **T**TG CTG TTG TGC CTG CC3' Arg Leu Leu Leu Leu **Leu** Leu Leu Cys Leu	**Val→Leu** **V L**

R L L L L **L** L L C L Binds 137-166bp of CD55 DNA sequence	32mer TM=84°C
CD55 Z69 Reverse Primer 5'GG CAG GCA CAA CAG CA**A** CAG CAG CAG CAG CCG3' Binds 137-166bp of CD55 DNA sequence	32mer TM=84°C
CD55 Z158m Forward Primer 5'CA GAT GTA CCT AAT G**T**C CAG CCA GCT TTG GAA GGC3' Asp Val Pro Asn **Val** Gln Pro Ala Leu Glu Gly D V P N **V** Q P A L E G Binds 198-232bp of CD55 DNA sequence	**Ala→Val** **A V** 35mer TM=80.13°C
CD55 Z158m Reverse Primer 5'GCC TTC CAA AGC TGG CTG G**A**C ATT AGG TAC ATC TG3' Binds 198-232bp of CD55 DNA sequence	35mer TM=80.13°C

Binding positions locate to annealing positions on DNA sequence accession number BC001288 (**Figure 2.2**). Highlighted bases indicate substitutions required to mutate the wild type sequence.

DNA constructs produced for immunisation were:

- SCR 1-3 Z143 Fc
- SCR 1-3 Z69 Fc
- SCR 1-3 Z143 Z69 Fc
- SCR 1-3 Z158m Fc
- SCR 1-3 Z143 Z158m Fc

Immunisation strategy

DNA micro-carriers were prepared as per **section 2.1.5** and male and female HLA-A*0201/k^b (HHDII) mice were immunised as per **section 2.1.6** at weekly intervals for a total of three immunisations. One week post final immunisation, animals underwent schedule1 protocol as per Home Office guidelines and splenocytes and sera were harvested.

Assessment of responses stimulated by DNA vaccine immunisations

Both humoral (**Chapter 4)** and cellular responses to the DNA constructs were assessed. This chapter incorporates results generated from cytotoxic T cell assays, ELISPOT assessment of cytokine release, and luminex analysis of cytokines present within sera and supernatant collected from *In Vitro* stimulated effector cells. The CTL assays required appropriate targets for obtained effector cells. Ideally, targets will naturally express HLA-A2 MHC and complete CD55 allowing for natural processing of target epitopes. Several cell lines used within the laboratory were analysed for this co-expression of desired molecules.

5.5: Assessment of cellular responses to the DNA vaccines

Cytotoxic T cell assays were chosen as one of the main strategies for the assessment of vaccine induced immune responses. Therefore a viable target cell was required expressing both human CD55 and HLA-A2 molecules. FACS analysis was carried out using indirect staining (**sections 2.2.8** to **2.2.10**) on several cell lines currently stored within the department. Of the four lines analysed, human tumour lines 791T and MKN45 both expressed high levels of CD55 (**Figure 5.11**) although both were

negative for HLA-A2. Colo 205 cells however, naturally expressed HLA-A2 but were negative for human CD55.

Figure 5.11: Cell lines assessed by flow cytometry for CD55 and HLA-A2 expression

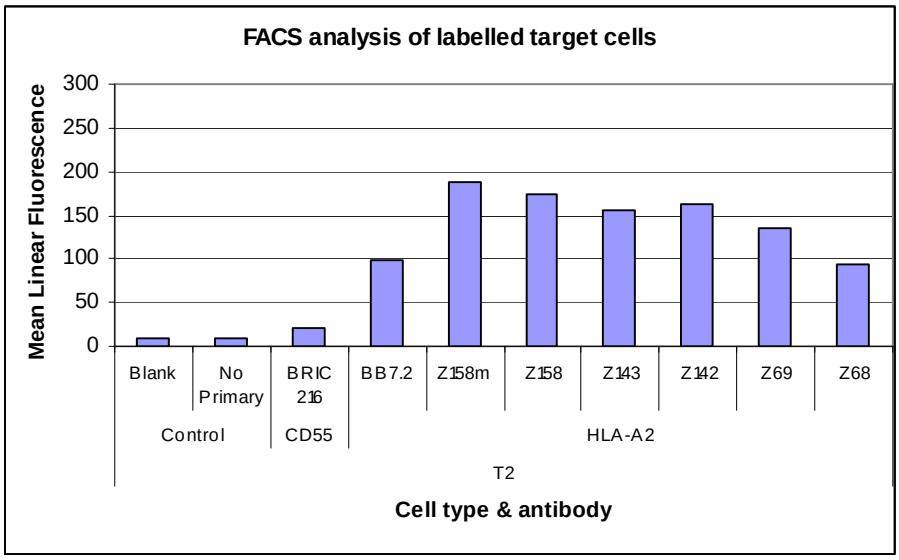
Standard analysis of CD55 and HLA-A2 expression (**Sections 2.2.8** to **22.2.10**) was carried out labelling 1 x 10^5 cells with 100µl of 5µg/ml primary antibody (CD55 and HLA-A2 specific) and subsequently with 400µl of 1/400 FITC conjugated rabbit anti mouse antibodies. Cells were washed in PBS and 5000 cells per sample were assessed by flow cytometry. Cells were gated using control samples labelled with secondary antibody alone and with unlabelled cells.

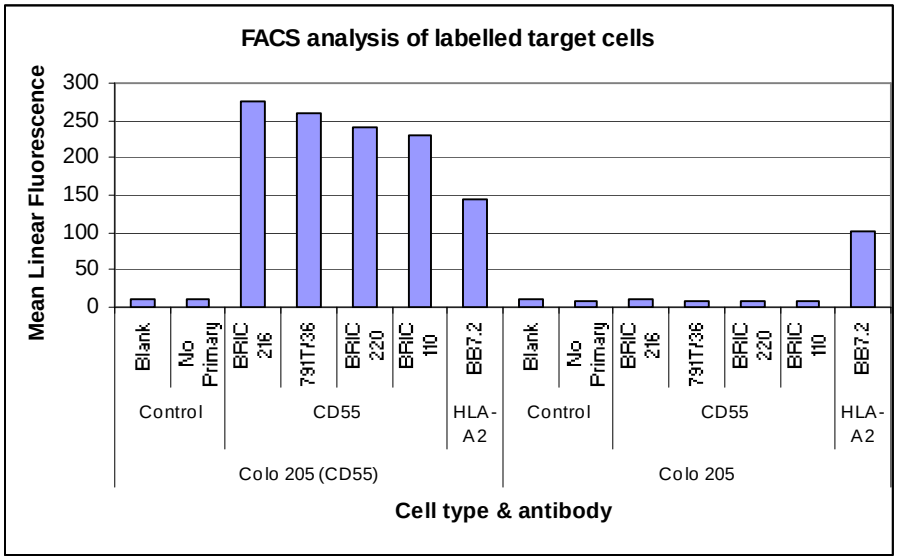
Cell Line	**Mean Liner Fluorescence**	
	CD55	**HLA-A2**
791T (human Osteosarcoma)	259	12.10
MKN45 (human gastric carcinoma)	262.2	9.1
Colo 205 (human colon adenocarcinoma)	11.2	107.194
T2 (Human lymphoblast)	12.2	97.2

As a viable target line was required, Colo 205 cells were transduced with a retrovirus incorporating full length human CD55 (see sections **2.2.7** to **2.2.9** and **2.2.11**). Colo 205 CD55 cells were then cultured under antibiotic selection conditions and a population of cells, with high expression levels, was isolated using FACS. **Figure 5.12a/b** shows the results for a standard binding assay for labelled Colo 205, transduced Colo 205 and T2 cells incubated with chosen peptides at 25µg/ml. Graph **a** enables comparison of T2 cells under varying conditions, and as can be seen these cells are CD55 negative and give an M.L.F for HLA-A2 of 100 and upon addition of stabilising peptides this value is increased to almost 200, with the greatest level observed with Z158m peptide. **Figure 5.12b** allows evaluation of Colo 205 and Colo 205 transduced to express human CD55. The transduced cells clearly show relatively high levels of CD55 expression. Antibodies specific to SCR domains 1, 2 and 3 were utilised to confirm expression of the complete CD55 structure and all generated M.L.F. values in excess of 225. HLA-A2 expression also appears elevated in the transduced cells in comparison to levels observed in the native Colo 205 cells.

Figure 5.12a/b: CD55 and HLA-A2 expression of T2, Colo 205 and Colo 205 CD55 cells

1 x 10^5 cells were labelled with CD55 and HLA-A2 specific primary antibodies as per **figure 5.11** and were stained with FITC conjugated secondary antibodies. 5000 cells were counted per sample and relative linear fluorescence was plotted.





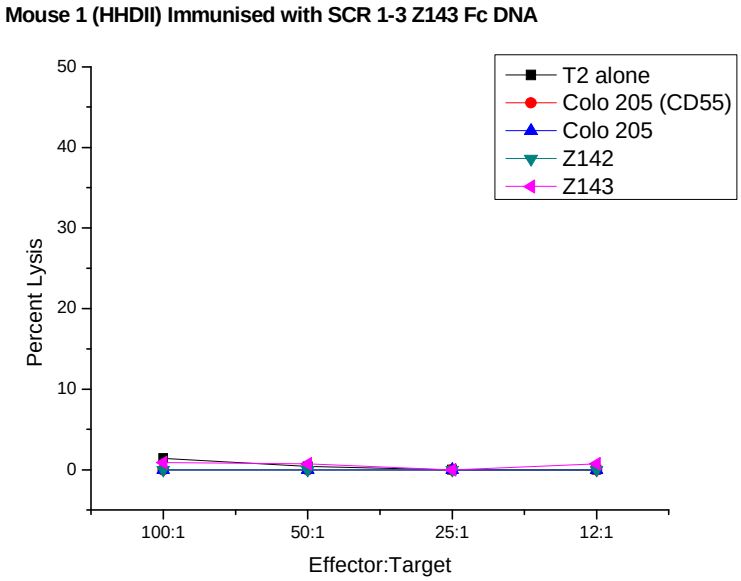
Several groups of immunisations were carried out on male and female HLA-A*0201/K^b mice that were between six and twelve weeks of age. DNA was coated onto 1.0µ gold particles and administered by the Helios Gene Gun (see **Sections 2.1.3** to **2.1.6**), with mice being immunised at weeks 1, 2 and 3 and spleens were removed and cells harvested at week 4. Both CTL (**Sections 2.7.1** to **2.7.4**) and ELISPOT (**Section 2.7.5**) assays were carried out on the harvested splenocytes for all groups. **Figure 5.13** displays the results obtained for SCR 1-3 Z143 Fc DNA immunisation.

Two groups of four mice were immunised and the results assessed with only group 2 results being shown. None of the four mice appear to raise a CTL response capable of eliciting cell lysis of either peptide pulsed T2 targets or Colo 205 CD55 cells with the greatest percentage lysis being 4%, observed at an effector: target ratio of 100:1 with no variation across the different targets. ELISPOT analysis of the same effectors assessing for antigen specific IFNγ release support the CTL findings, showing that for 10^6 cells seeded only 60 spot forming units were developed for one mouse specific to the wild type peptide Z142.

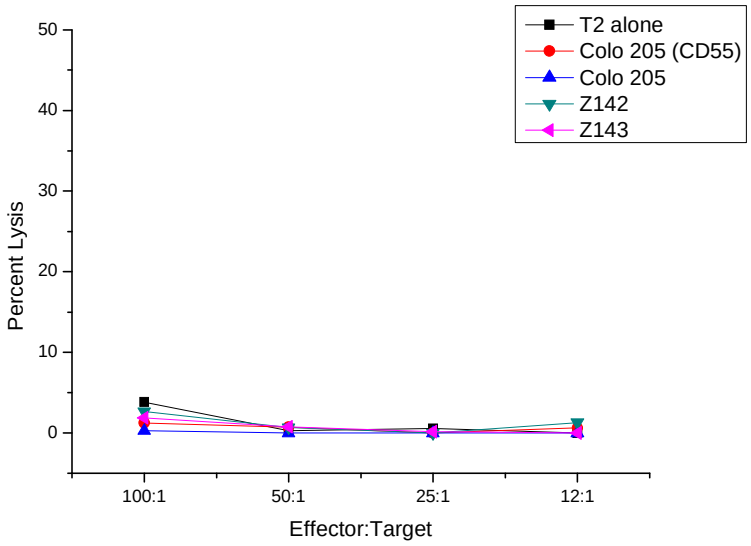
Figure 5.13: Immunisation of HHD II mice with SCR 1-3 Z143 Fc DNA

Figure 5.13a: Cytotoxic T Cell (CTL) Assay

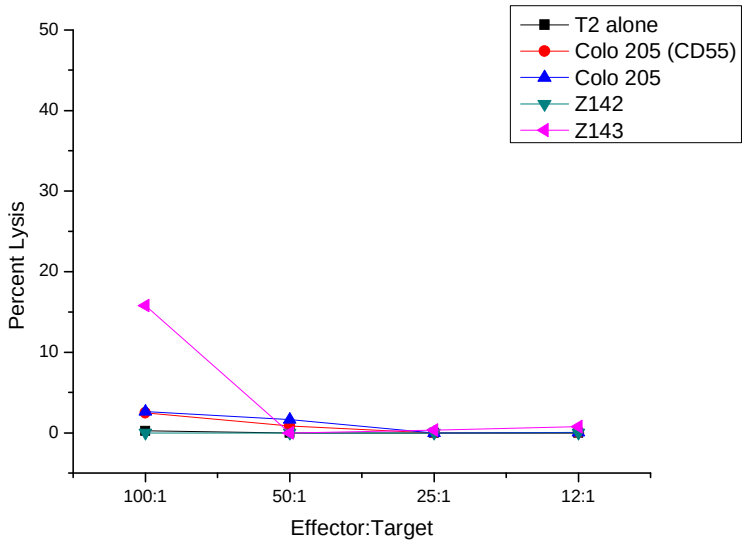
Standard assay protocol was followed as per **sections 2.7.1** to **2.7.4**. Target cells were labelled with 1.85MBq sodium [^{51}Cr] chromate for 1 hour with or without 100µg/ml specific peptide, washed and repeat labelled with or without specific peptide for a further hour. 5000 cells were seeded into 96 U well plates and co cultured with titrated numbers of effector cells in an assay volume of 200µl of complete media. Prior to the assay, effector cells had been co cultured *In Vitro* with 1 x 10^6 irradiated LPS blasts which had previously been incubated with 10µg/ml target peptides. Plates were incubated at 37°C 5% CO_2 for 4 hours and 50µl of assay culture media was transferred to lumaplates. Plates were allowed to air dry and were then analysed on a scintillation counter. Percent lysis was calculated relative to spontaneous and maximum lysis results.



Mouse 2 (HHDII) Immunised with SCR 1-3 Z143 Fc DNA



Mouse 3 (HHDII) Immunised with SCR 1-3 Z143 Fc DNA



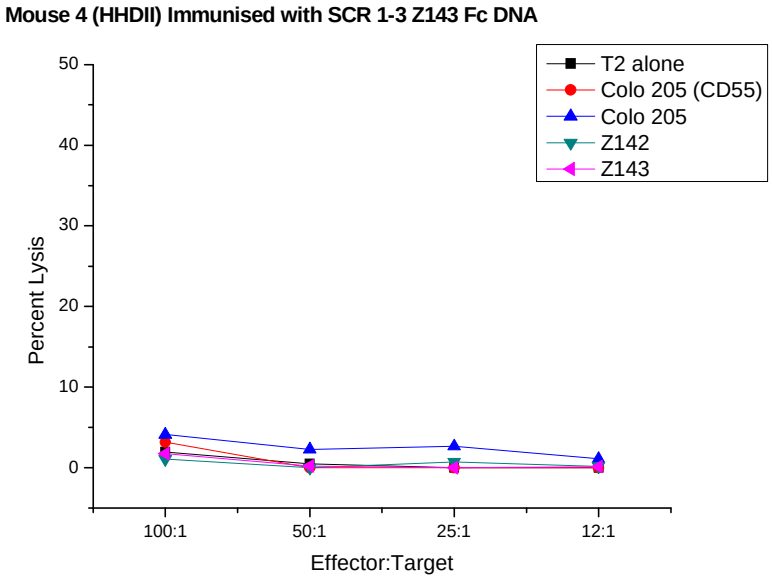
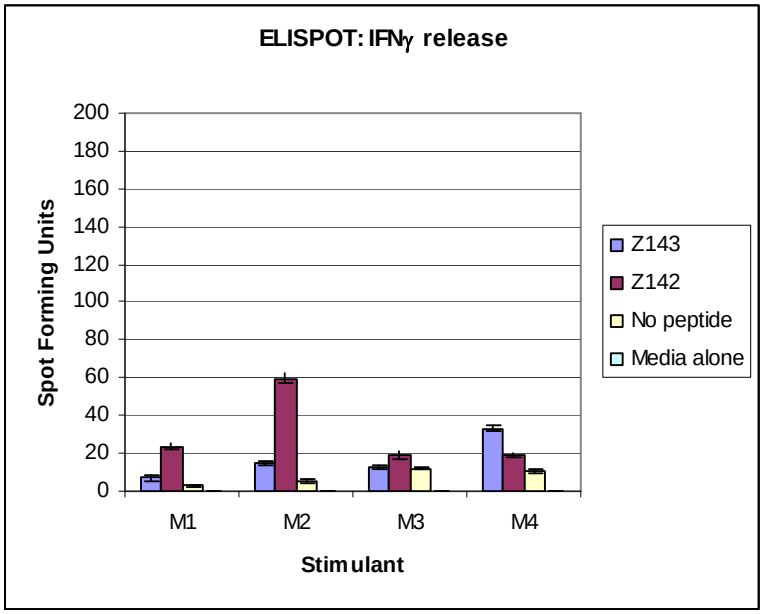


Figure 1.13b: ELISPOT analysis of Interferon γ production

Standard protocol was followed as per **section 2.7.5** utilising the ELISPOT kit from R & D Systems. 96 well assay plates were coated with cytokine specific capture antibody overnight at 4°C. Non specific binding sites were blocked and 1 x 10^6 effector cells were seeded per well. Splenocytes were then stimulated with and without 10µg/ml target peptide and control wells were set up containing media alone, cells alone and wells containing cells with PMA and Ionomycin as a positive control to test viability of cell samples. All conditions were repeated in triplicate and plates were incubated at 37°C 5% CO_2 for 16 hours. Cells were then removed and cytokine specific capture antibody was added to all wells. The development module from the kit was then used for development of the assay prior to analysis on a Bioreader 3000 Pro. Spots were counted and results expressed as the number of spot forming units [S.F.U (cells)] that produced cytokine in response to specific stimuli.



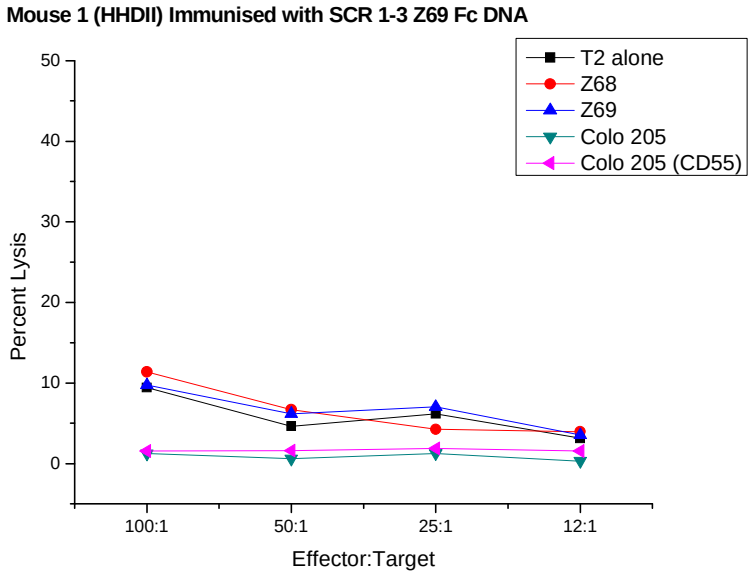
Results shown in S.F.U per 10^6 cells for all mice immunised from the same group.

Figure 5.14 displays results obtained for SCR 1-3 Z69 Fc DNA immunisation. Two groups of four were assessed and results were consistent across both groups. Results are shown for one of the groups and indicate that a limited CTL response is generated specific for either peptide pulsed targets or the COLO 205 CD55 cells. 10% lysis is observed for three out of our mice at 100:1 effector: target ratio but non-specifically as responses to Z68, Z69 and T2 cells without peptide are similar. No lysis is observed for natively expressed CD55 when compared with Colo 205 $CD55^{-/-}$ cells. ELISPOT assessment of IFNγ release for the same effectors supports CTL evidence of limited to no response, as less than 5 spot forming units per 10^6 cells are generated in response to specific peptides.

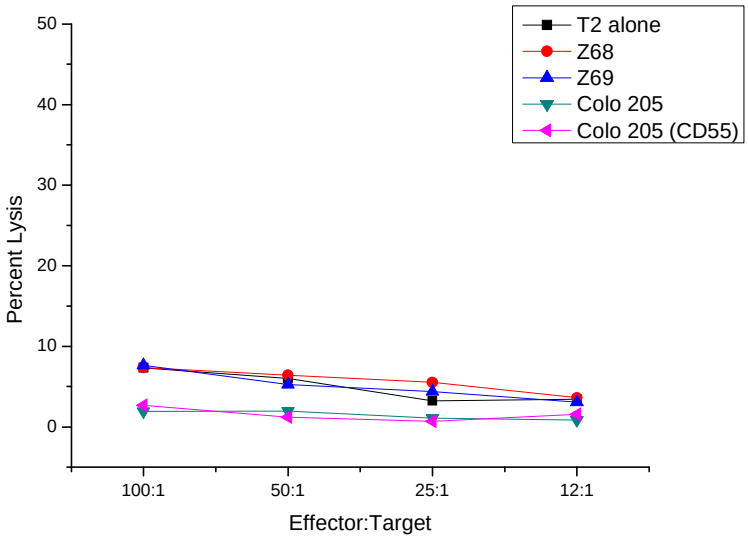
Figure 5.14: Immunisation of HHD II mice with SCR 1-3 Z69 Fc DNA

Figure 5.14a: Cytotoxic T Cell (CTL) Assay

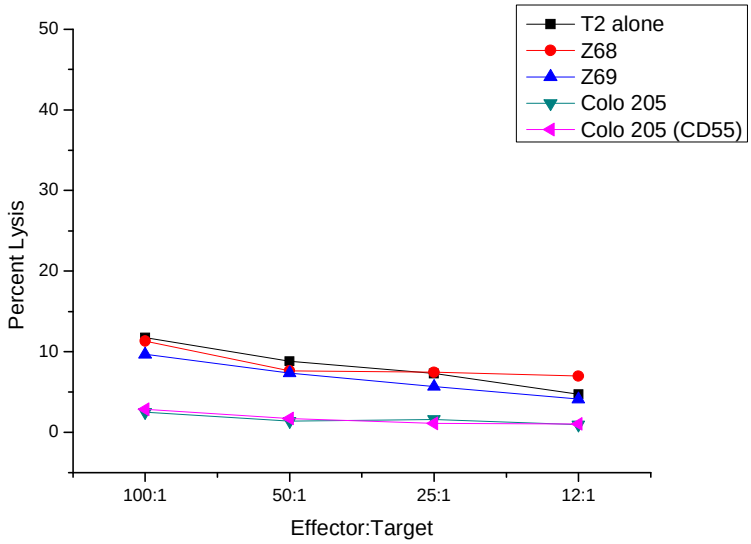
Standard assay protocol was followed as per **sections 2.7.1** to **2.7.4**. Target cells were labelled with 1.85MBq sodium [^{51}Cr] chromate for 1 hour with or without 100µg/ml specific peptide, washed and repeat labelled with or without specific peptide for a further hour. 5000 cells were seeded into 96 U well plates and co cultured with titrated numbers of effector cells in an assay volume of 200µl of complete media. Prior to the assay, effector cells had been co cultured *In Vitro* with 1 x 10^6 irradiated LPS blasts which had previously been incubated with 10µg/ml target peptides. Plates were incubated at 37°C 5% CO_2 for 4 hours and 50µl of assay culture media was transferred to lumaplates. Plates were allowed to air dry and were then analysed on a scintillation counter. Percent lysis was calculated relative to spontaneous and maximum lysis results.



Mouse 2 (HHDII) Immunised with SCR 1-3 Z69 Fc DNA



Mouse 3 (HHDII) Immunised with SCR 1-3 Z69 Fc DNA



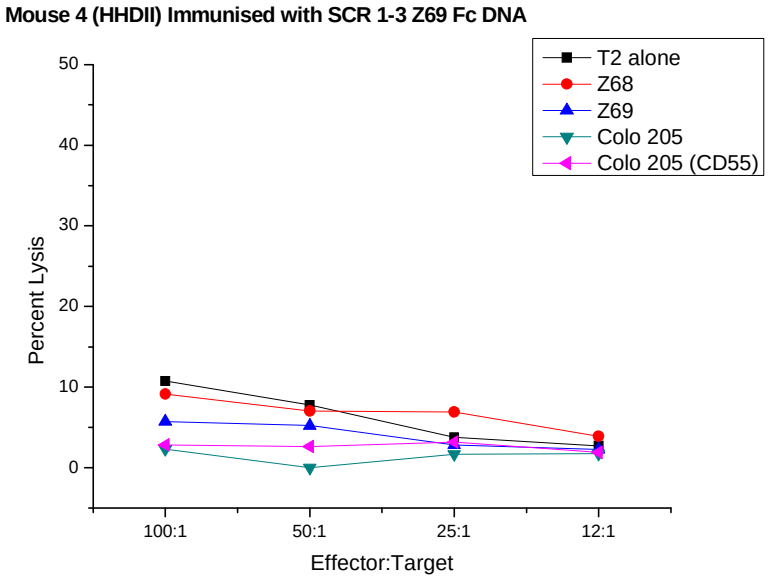
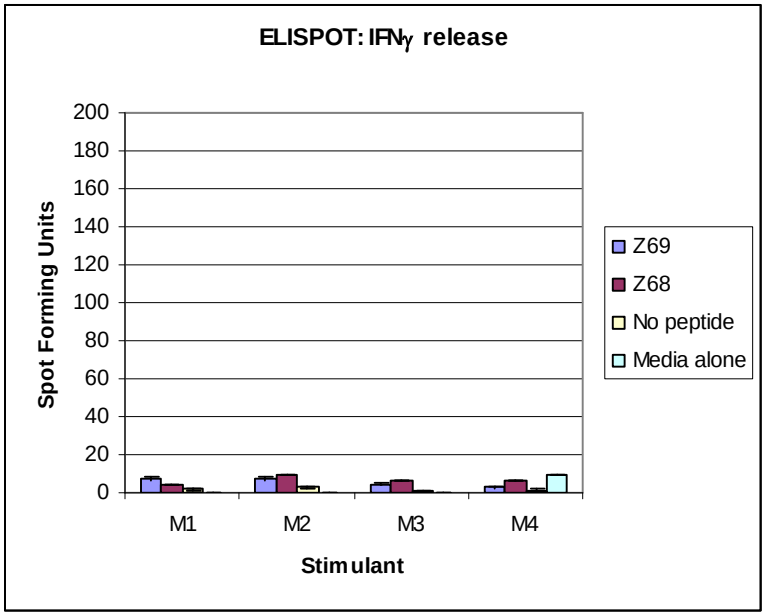


Figure 1.14b: ELISPOT analysis of Interferon γ production

Standard protocol was followed as per **section 2.7.5** utilising the ELISPOT kit from R & D Systems. 96 well assay plates were coated with cytokine specific capture antibody overnight at 4°C. Non specific binding sites were blocked and 1 x 10^6 effector cells were seeded per well. Splenocytes were then stimulated with and without 10µg/ml target peptide and control wells were set up containing media alone, cells alone and wells containing cells with PMA and Ionomycin as a positive control to test viability of cell samples. All conditions were repeated in triplicate and plates were incubated at 37°C 5% CO_2 for 16 hours. Cells were then removed and cytokine specific capture antibody was added to all wells. The development module from the kit was then used for development of the assay prior to analysis on a Bioreader 3000 Pro. Spots were counted and results expressed as the number of spot forming units [S.F.U (cells)] that produced cytokine in response to specific stimuli.



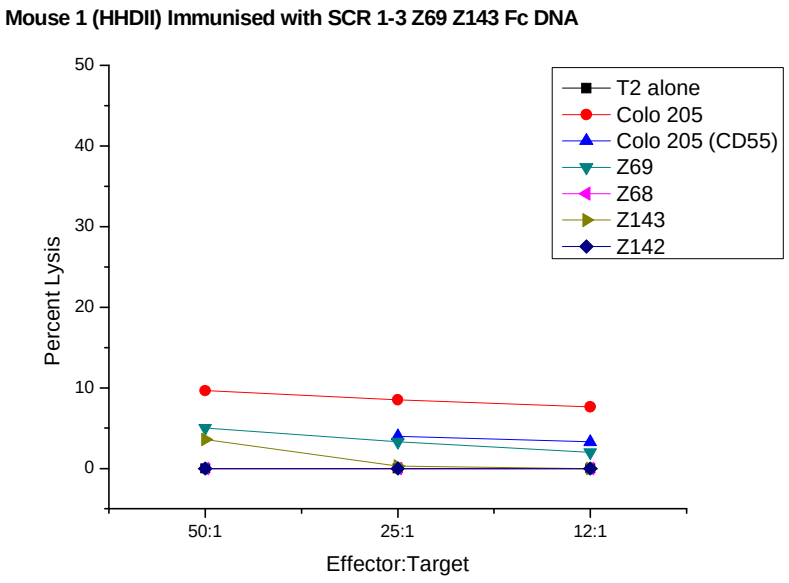
Results shown in S.F.U per 10^6 cells for all mice immunised from the same group.

Compound vaccines were also created incorporating both Z69 and Z143 and **Figure 5.15** displays the CTL and IFNγ ELISPOT results obtained for this group. One group of four mice were immunised and splenocytes from only one mouse generated killing of any targets. Maximum lysis was calculated at 10% at a 50:1 effector: target ratio, but again this appears to be a non-specific response as T2 cells alone and Colo 205 cell targets generated equal lysis. ELISPOT analysis for IFNγ release also confirms that minimal antigen specific responses were stimulated by this construct as only mouse 4 shows 70 spot forming units +/- 10 stimulated by the wild type peptide Z142 with less than 30 obtained for other stimuli.

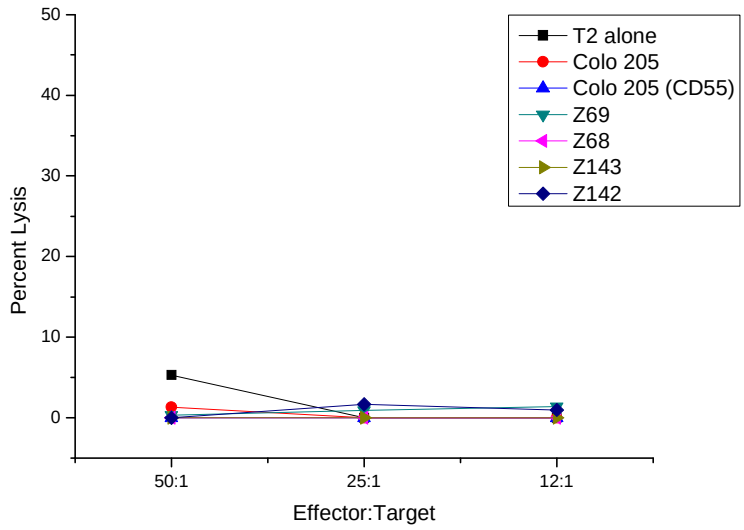
Figure 5.15: Immunisation of HHD II mice with SCR 1-3 Z69 Z143 Fc DNA

Figure 5.15a: Cytotoxic T Cell (CTL) Assay

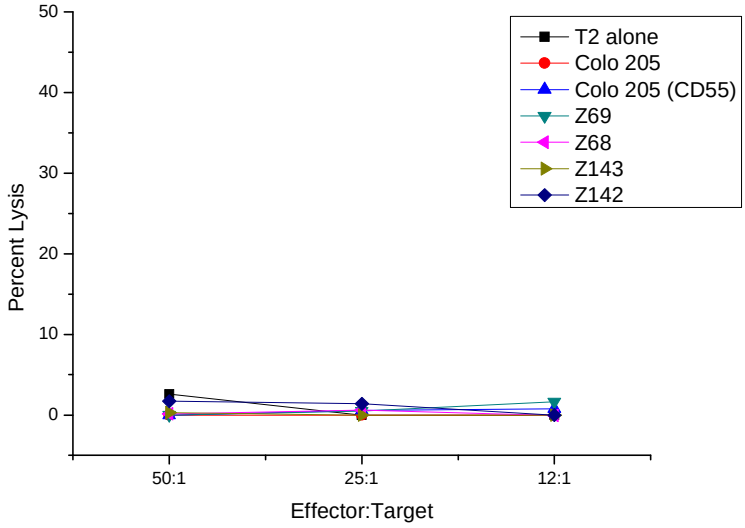
Standard assay protocol was followed as per **sections 2.7.1** to **2.7.4**. Target cells were labelled with 1.85MBq sodium [^{51}Cr] chromate for 1 hour with or without 100µg/ml specific peptide, washed and repeat labelled with or without specific peptide for a further hour. 5000 cells were seeded into 96 U well plates and co cultured with titrated numbers of effector cells in an assay volume of 200µl of complete media. Prior to the assay, effector cells had been co cultured *In Vitro* with 1 x 10^6 irradiated LPS blasts which had previously been incubated with 10µg/ml target peptides. Plates were incubated at 37°C 5% CO_2 for 4 hours and 50µl of assay culture media was transferred to lumaplates. Plates were allowed to air dry and were then analysed on a scintillation counter. Percent lysis was calculated relative to spontaneous and maximum lysis results.



Mouse 2 (HHDII) Immunised with SCR 1-3 Z69 Z143 Fc DNA



Mouse 3 (HHDII) Immunised with SCR 1-3 Z69 Z143 Fc DNA



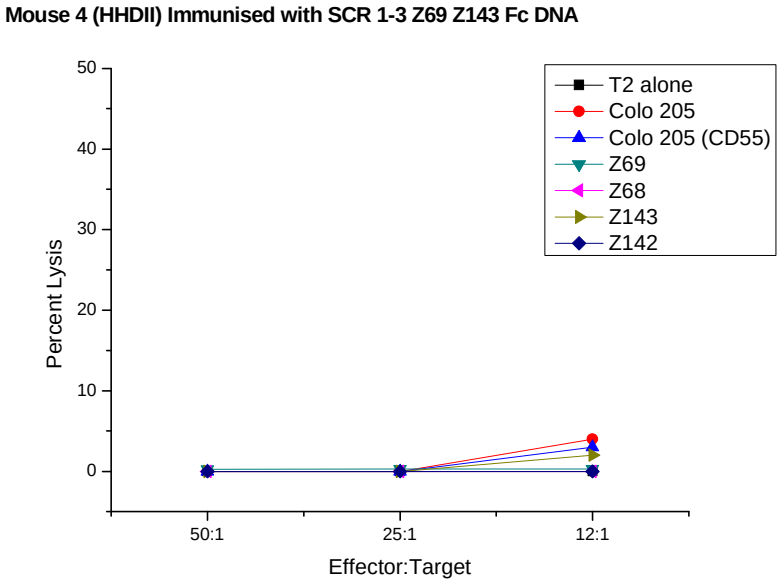
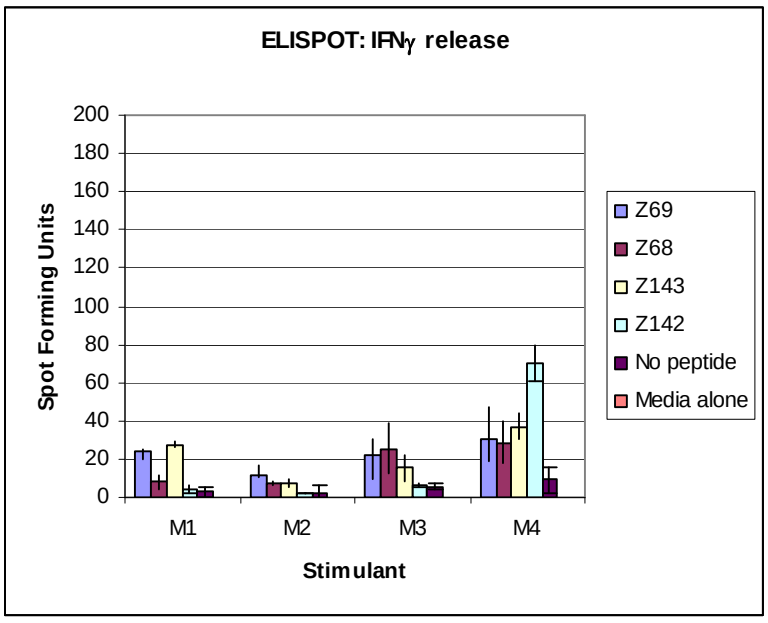


Figure 5.15b: ELISPOT analysis of Interferon γ production

Standard protocol was followed as per **section 2.7.5** utilising the ELISPOT kit from R & D Systems. 96 well assay plates were coated with cytokine specific capture antibody overnight at 4°C. Non specific binding sites were blocked and 1 x 10^6 effector cells were seeded per well. Splenocytes were then stimulated with and without 10µg/ml target peptide and control wells were set up containing media alone, cells alone and wells containing cells with PMA and Ionomycin as a positive control to test viability of cell samples. All conditions were repeated in triplicate and plates were incubated at 37°C 5% CO_2 for 16 hours. Cells were then removed and cytokine specific capture antibody was added to all wells. The development module from the kit was then used for development of the assay prior to analysis on a Bioreader 3000 Pro. Spots were counted and results expressed as the number of spot forming units [S.F.U (cells)] that produced cytokine in response to specific stimuli.



Results shown in S.F.U per 10^6 cells for all mice immunised from the same group.

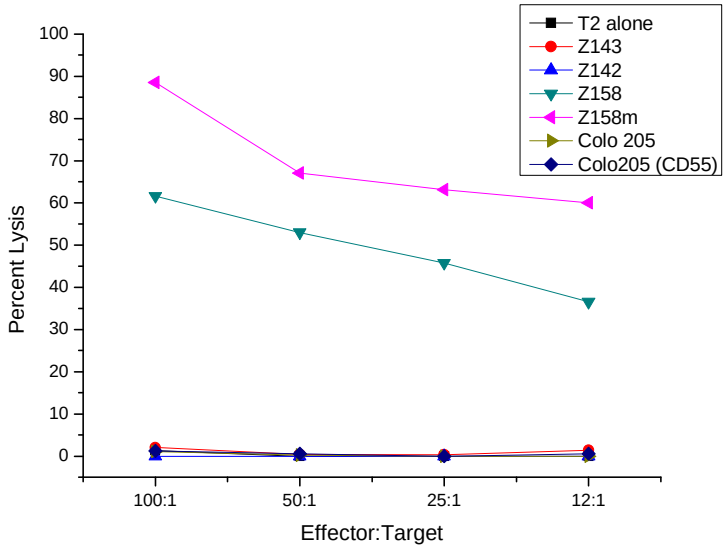
Figure 5.16 shows results for another double mutant construct containing both Z143 and Z158m within the SCR 1-3 Fc DNA vaccine. CTL analysis of the double construct immunisation indicate that significant levels of antigen specific lysis has been stimulated for all four mice with lysis in ranging from 90% to 50% at an effector: target ratio of 100:1 for Z158m peptide pulsed targets. The CTLs generated also effectively kill wild type peptide (Z158) pulsed targets at a range between 60% and 40% at the same E:T ratio. Antigen specific lysis of both Z158m and Z158 pulsed targets remains consistent through to E:T ratio of 12:1 ranging between 60% and 30%. However, lysis of either Z143 or Z142 pulsed targets remains below 5%, equivalent with background levels of lysis for alternate targets including Colo 205 CD55 and Colo 205 cells. ELISPOT assessment for IFNγ support these findings with both mouse 1 and 3 generating the greatest level of antigen specific responses, specifically to Z158m and its wild type counterpart. Per 10^6 cells seeded S.F.U counts were in excess of 350 and in some wells were too dense to count. Interestingly, IFNγ production was also seen in responses to Z142 *in vitro* stimulation although this was only observed in cells from mice which generated the greatest responses to both Z158m and Z158.

Figure 5.16: Immunisation of HHD II mice with SCR 1-3 Z143 Z158m Fc DNA

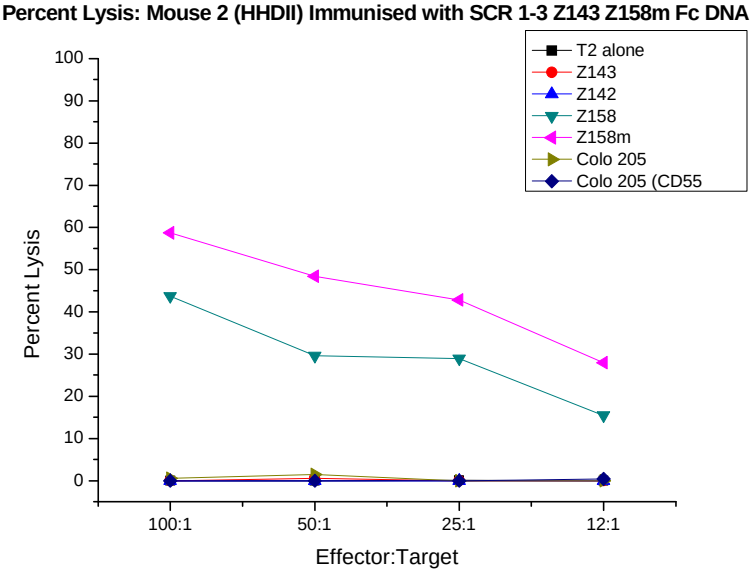
Figure 5.16a: Cytotoxic T Cell (CTL) Assay

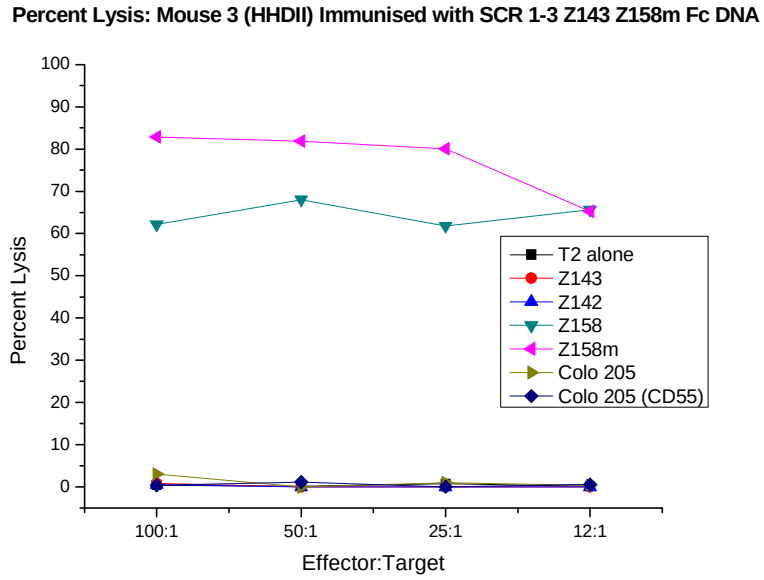
Standard assay protocol was followed as per **sections 2.7.1** to **2.7.4**. Target cells were labelled with 1.85MBq sodium [^{51}Cr] chromate for 1 hour with or without 100µg/ml specific peptide, washed and repeat labelled with or without specific peptide for a further hour. 5000 cells were seeded into 96 U well plates and co cultured with titrated numbers of effector cells in an assay volume of 200µl of complete media. Prior to the assay, effector cells had been co cultured *In Vitro* with 1 x 10^6 irradiated LPS blasts which had previously been incubated with 10µg/ml target peptides. Plates were incubated at 37°C 5% CO_2 for 4 hours and 50µl of assay culture media was transferred to lumaplates. Plates were allowed to air dry and were then analysed on a scintillation counter. Percent lysis was calculated relative to spontaneous and maximum lysis results.

Percent Lysis: Mouse 1 (HHDII) Immunised with SCR 1-3 Z143 Z158m Fc DNA



Percent Lysis: Mouse 2 (HHDII) Immunised with SCR 1-3 Z143 Z158m Fc DNA





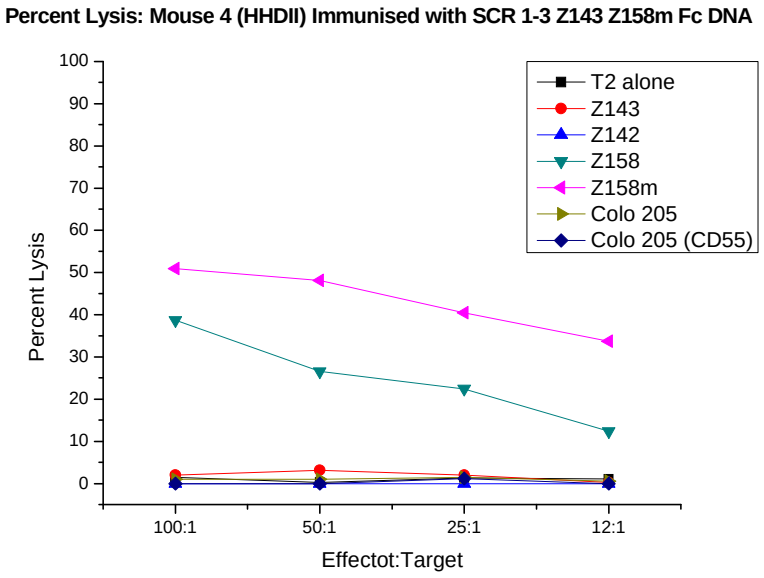
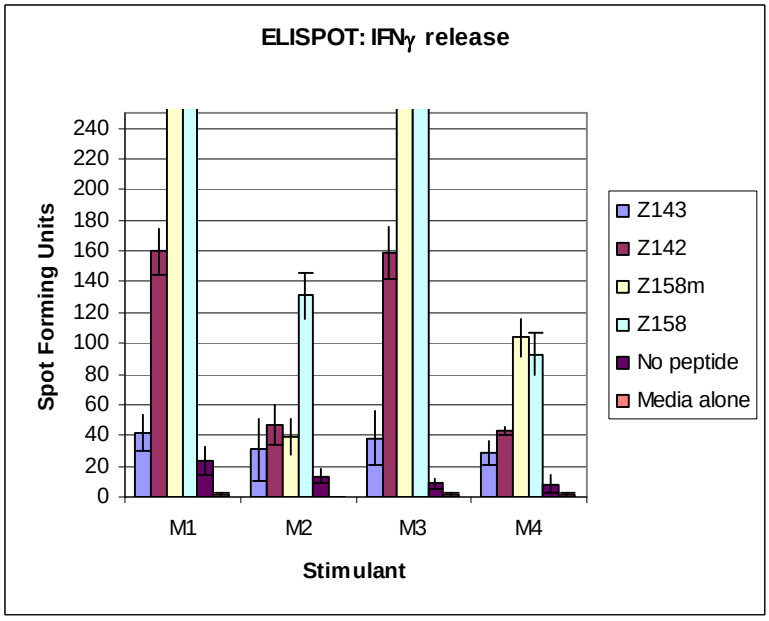


Figure 1.16b: ELISPOT analysis of Interferon γ production

Standard protocol was followed as per **section 2.7.5** utilising the ELISPOT kit from R & D Systems. 96 well assay plates were coated with cytokine specific capture antibody overnight at 4°C. Non specific binding sites were blocked and 1 x 10^6 effector cells were seeded per well. Splenocytes were then stimulated with and without 10µg/ml target peptide and control wells were set up containing media alone, cells alone and wells containing cells with PMA and Ionomycin as a positive control to test viability of cell samples. All conditions were repeated in triplicate and plates were incubated at 37°C 5% CO_2 for 16 hours. Cells were then removed and cytokine specific capture antibody was added to all wells. The development module from the kit was then used for development of the assay prior to analysis on a Bioreader 3000 Pro. Spots were counted and results expressed as the number of spot forming units [S.F.U (cells)] that produced cytokine in response to specific stimuli.



Results shown in S.F.U per 10^6 cells for all mice immunised from the same group.

Due to the responses generated with the SCR 1-3 Z143 Z158m Fc DNA construct, two groups of mice were set up directly comparing this vaccine with the single epitope vaccine containing Z158m. **Figure 5.17** displays the results obtained for the double epitope construct assessing CTL mediated killing of Z158m and Z158 pulsed T2 targets and also Colo 205 CD55 and Colo 205 cells. ELIPOT analysis was carried out analysing both IFNγ and IL-4 release stimulated by the full complement of stimuli under two concentrations of effector cells namely 10^6 and 5 x 10^5. Mouse 1 elicited CTL killing of both Z158m and Z158 pulsed T2 cells ranging from 37% to 10% titrating down respective to effector cell concentration. 12% killing was also observed of Colo 205 CD55 cells in comparison with Colo 205 cell lysis which did not elevate above 2%. Antigen specific IFNγ release again supports this data, showing that responses are directed against Z158 and Z158m (>100 S.F.U) with similar frequency showing a titrated effect when assessed in the presence of 5 x 10^5 effector cells (75 S.F.U). Minimal responses were observed for the assessment of IL-4 release, generating in the range of 35 S.F.U in the presence of all stimuli. Z143 and Z142 were also screened in tandem again producing limited responses. Mouse 2 also elicited up 20% antigen specific killing of T2 cells pulsed with Z158 and Z158m at 100:1 E: T ratio with a titrated effect in response to reduced effector cell number. 5% lysis of Colo 205 CD55 cells was also observed at maximum effector cell density which was greater than the 1-2% observed for Colo 205 cells. These results may have

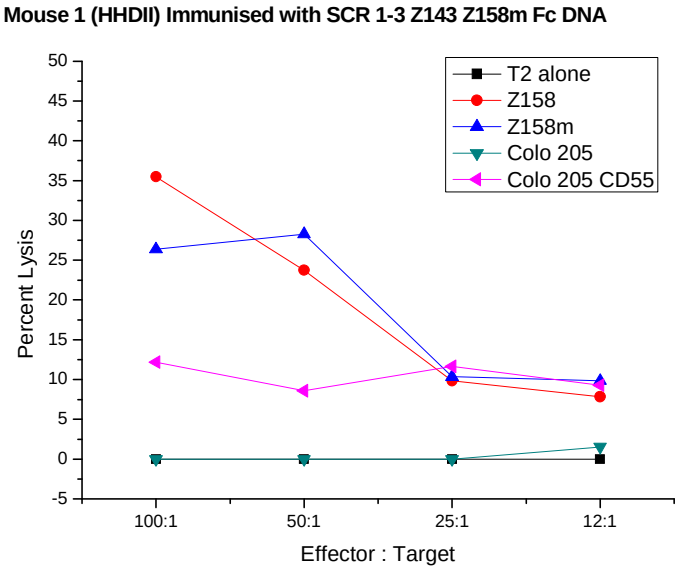
been lower than those for mouse 1, although ELISPOT analysis again showed IFNγ release for both the Z158 and Z158m stimulated cells with S.F.U. in excess of 100 for 10^6 seeded cells. This antigen specific cytokine release was also observed at effector concentration of 5 x 10^5 cells. Again Z143 and Z142 stimulated 60 S.F.U. which was only slightly greater than release for un-stimulated cells. Mouse 3 displayed similar results as mouse 2 generating between 15 and 20% lysis of Z158 and Z158m pulsed targets with 10% lysis of Colo 205 CD55 cells being observed compared to no killing of CD55 negative cells. IFNγ release supported the response to 158m and its wild type peptide with limited responses to Z143 and Z142 and little to no IL-4 responses for any test stimuli. Mouse 4 elicited strong CTL responses to Z158 and Z158m pulsed T2 cells generating between 40 and 50% lysis at an E: T of 100:1 dropping to 27% at a 12: 1 ratio. Colo 205 CD55 lysis was also generated at 10% at 100: 1 E: T ratio. This high level of antigen specific lysis was also supported by IFNγ release assessment with up to 200 S.F.U. generated for both Z158 and its mutant. Again limited IL-4 release was observed with no response to Z143 or Z142 for either cytokine. A general trend can be observed that while greater overall responses are directed towards the mutant peptides, as would be expected, significant responses are still generated towards the wild type sequence.

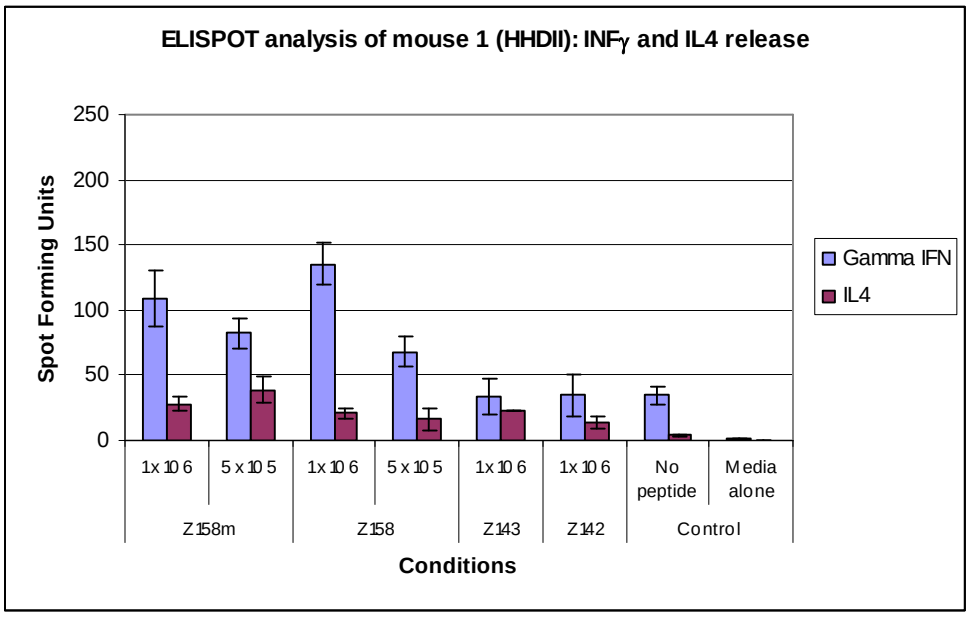
Figure 5.17: Immunisation of HHD II mice with SCR 1-3 Z143 Z158m Fc DNA

Repeat analysis of Z143 Z158 double construct as a direct comparison with single mutated epitope Z158 (experiment ran in tandem with **Figure 5.18**).

Figure 5.17a: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 1)

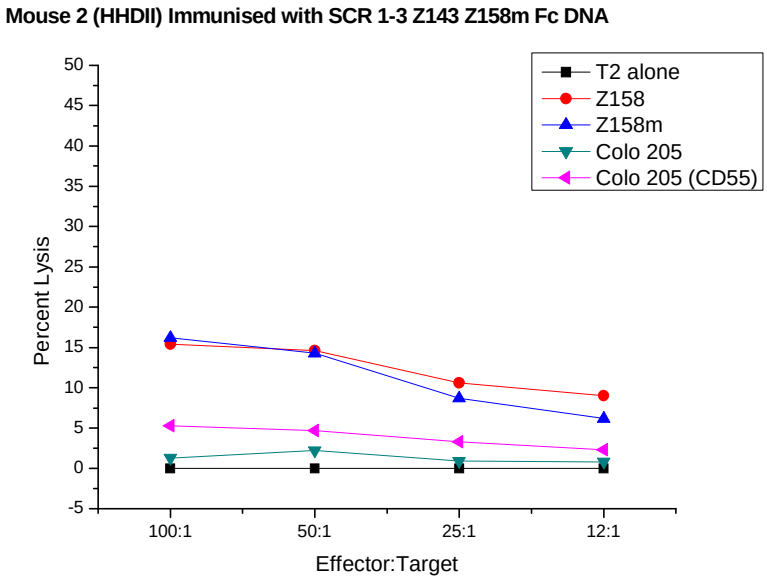
Standard protocol followed with assessment of only Z158m and Z158 peptides as previous analysis indicated no response generated towards Z142 mutant.

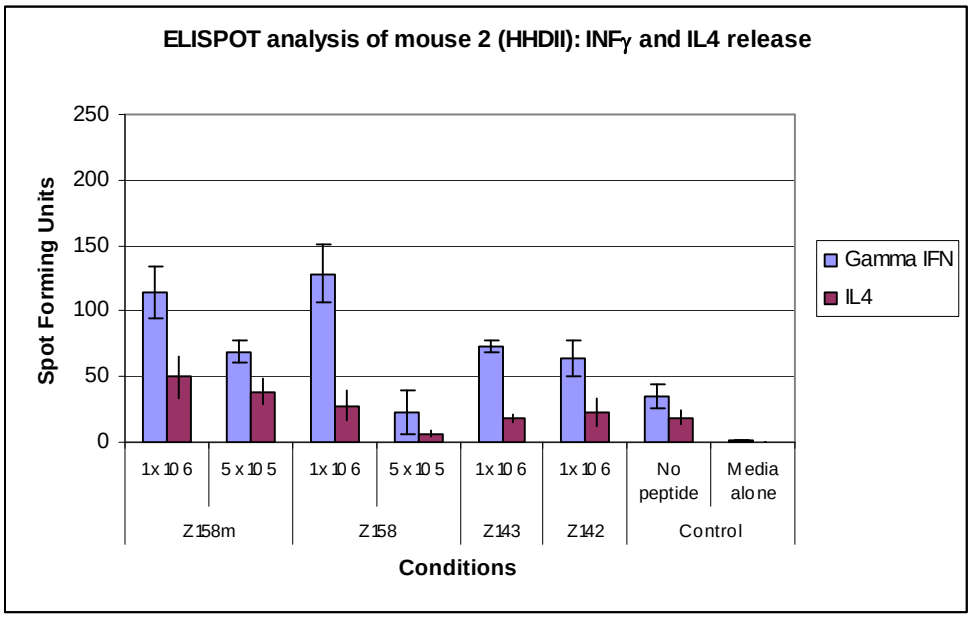




Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with both Z158 and Z142 wild type and mutant peptides.

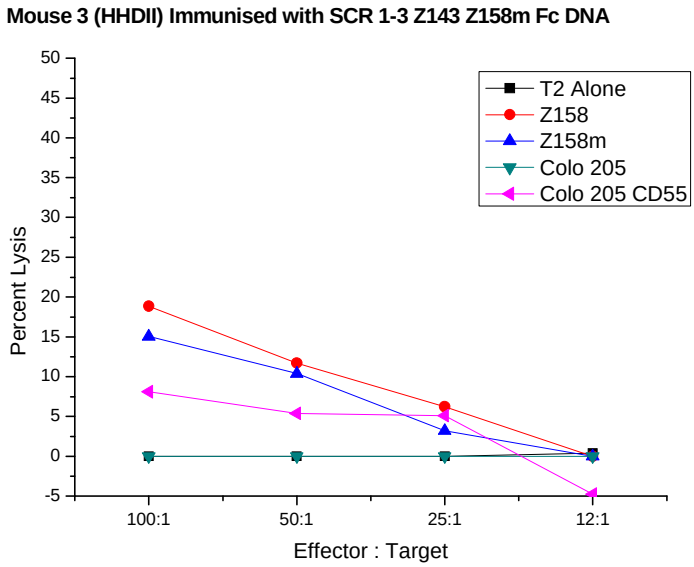
Figure 5.17b: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 2)

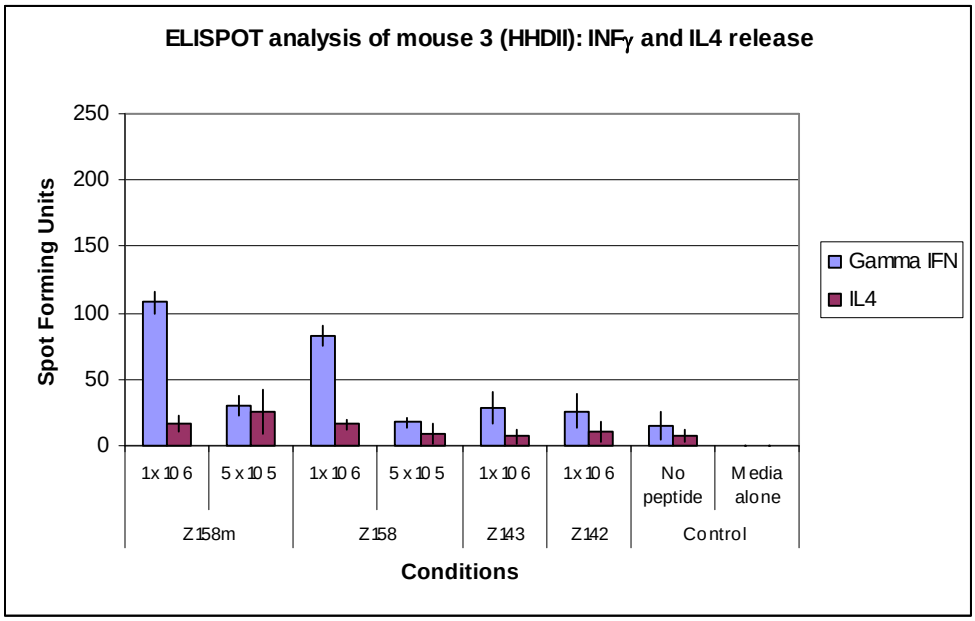




Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with both Z158 and Z142 wild type and mutant peptides.

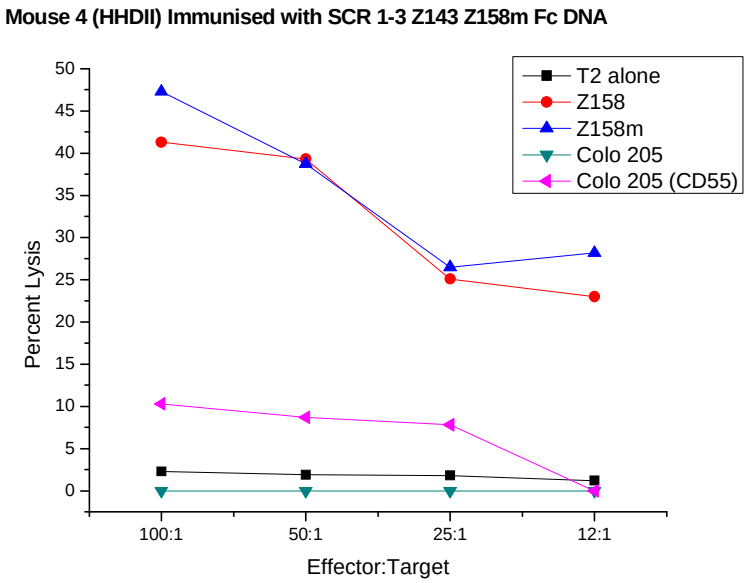
Figure 5.17c: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 3)

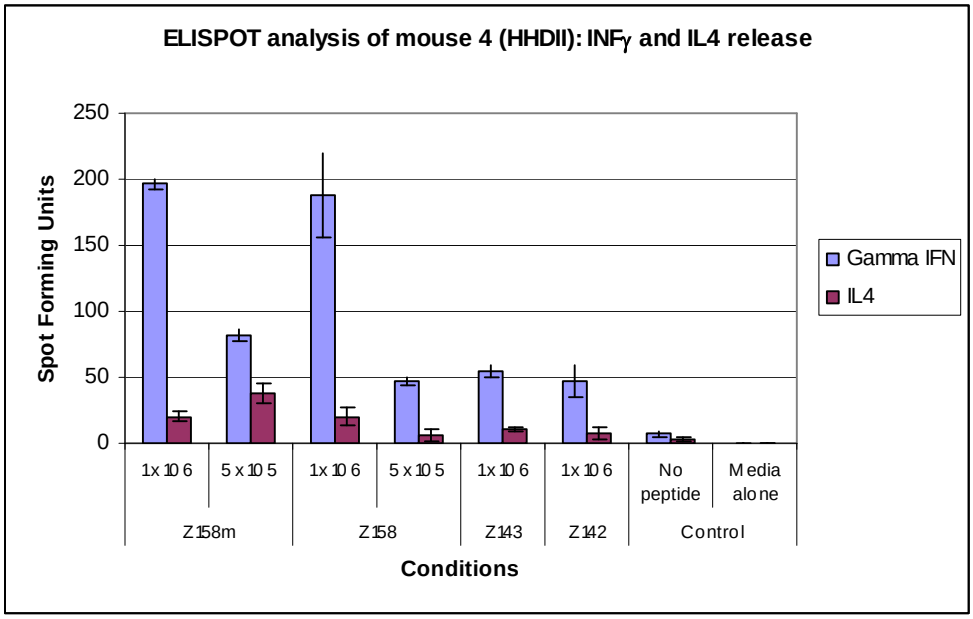




Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with both Z158 and Z142 wild type and mutant peptides.

Figure 5.17d: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 4)





Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with both Z158 and Z142 wild type and mutant peptides.

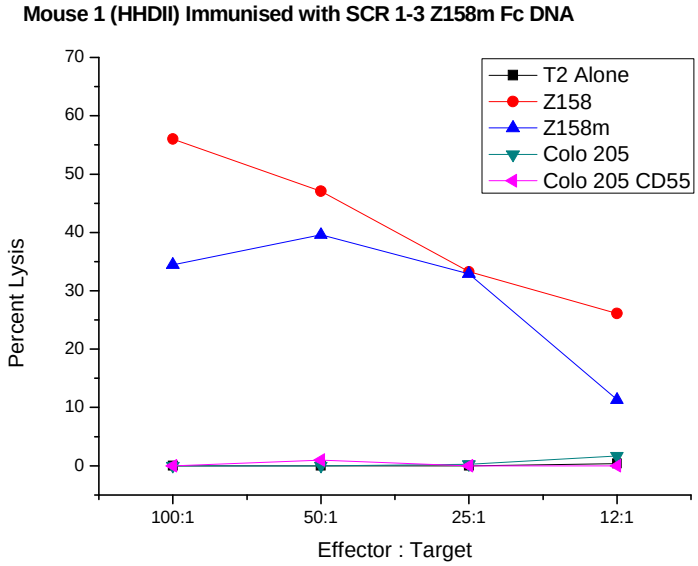
Figure 5.18 displays the results for a repeat SCR 1-3 Z158m Fc DNA immunisation ran in tandem with the double epitope vaccine. Three of the four mice generate CTL mediated lysis of Z158 and Z158m pulsed targets between 35% and 60% at 100:1 E: T ratio with limited to no response against the Colo 205 CD55 cells. Observed lysis

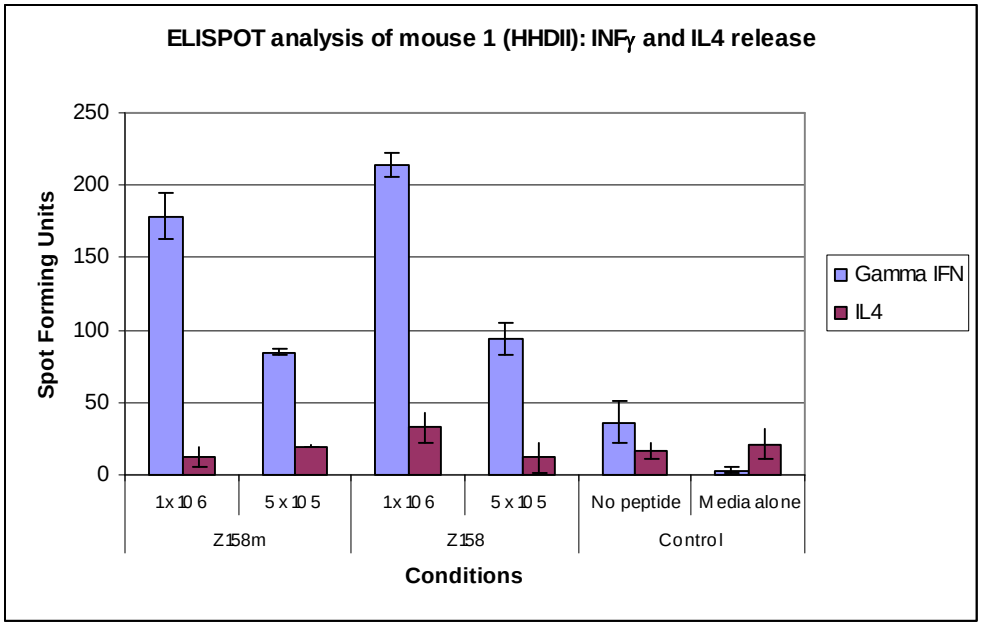
titrates with limiting effector cell concentration to approximately 15% at a 12:1 ratio. All three mice generate up to 200 S.F.U. when assessed for antigen specific IFNγ release. These responses are reduced when 5 x 10^5 effector cells are used with three out of four mice generating up to 100 S.F.U. IL-4 assessment again showed limited responses, generating approximately 40 S.F.U. being comparable to base line results. Mouse 4 showed the weakest of all responses with between 15% and 20% CTL mediated lysis of peptide pulsed targets, no killing of CD55 positive cells and IFNγ release displayed at 60 S.F.U with IL-4 release being limited to around 30 S.F.U.

Figure 5.18: Immunisation of HHD II mice with SCR 1-3 Z158m Fc DNA

Figure 5.18a: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 1)

Standard protocols followed for both CTL and ELISPOT assays.

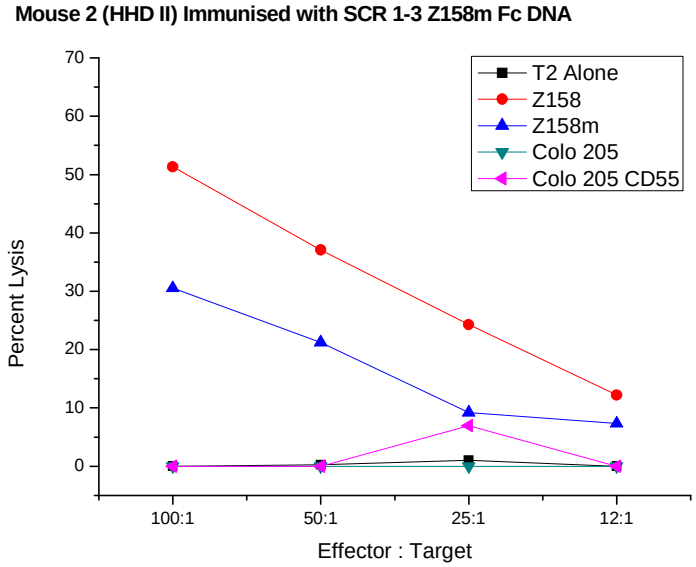


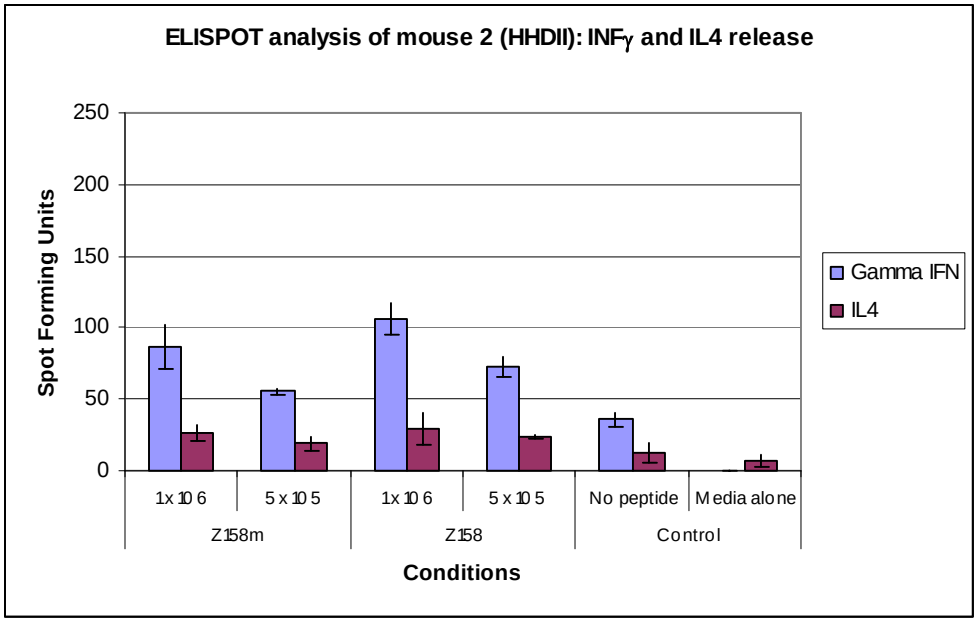


Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with Z158 wild type and mutant peptides.

Figure 5.18b: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 2)

Standard CTL protocol followed.

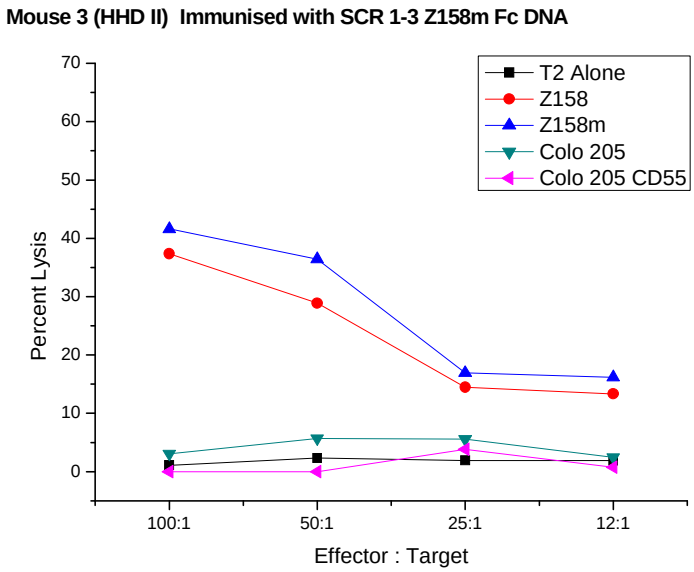


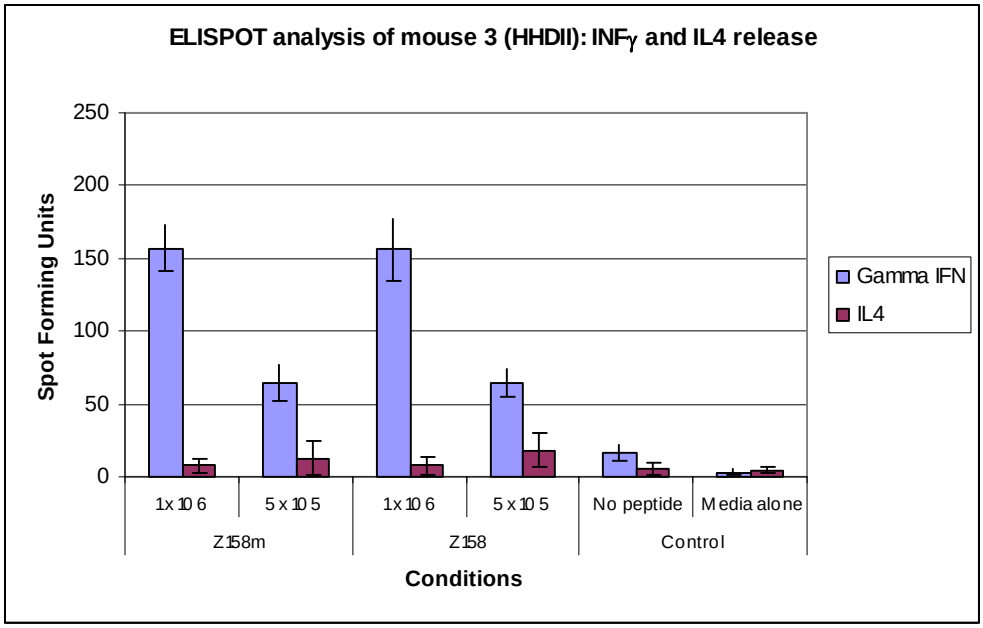


Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with Z158 wild type and mutant peptides.

Figure 5.18c: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 3)

Standard CTL protocol followed.

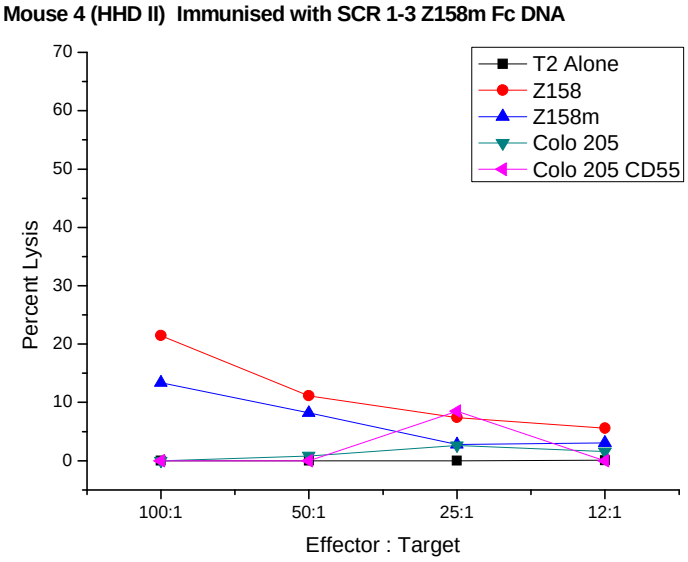


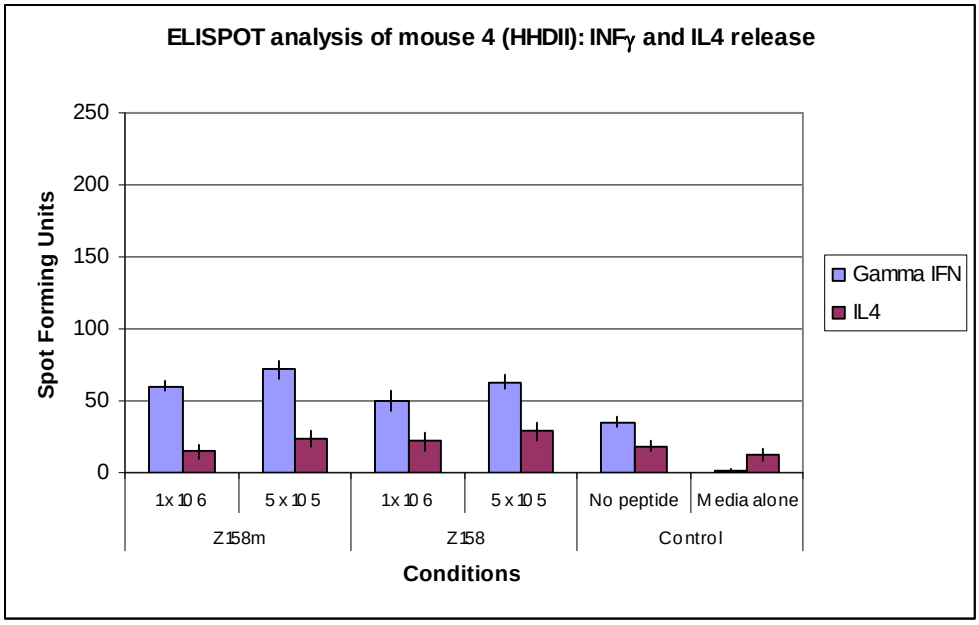


Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with Z158 wild type and mutant peptides.

Figure 5.18d: Cytotoxic T Cell (CTL) Assay and ELISPOT analysis of IFNγ and IL-4 antigen specific release (Mouse 4)

Standard CTL protocol followed.





Standard ELISPOT protocol followed utilising 1 x 10^6 and 5 x 10^5 effector cells seeded with Z158 wild type and mutant peptides.

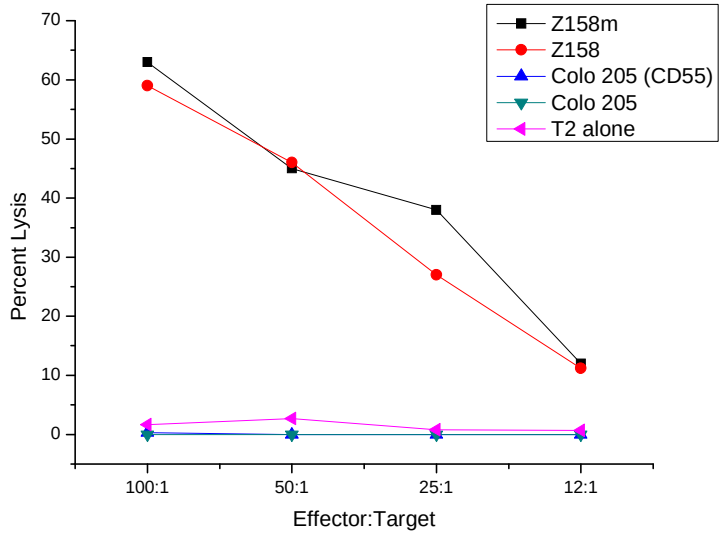
Repeat analysis of the SCR 1-3 Z158m Fc DNA vaccine was set up to confirm previous trends observed. **Figure 5.19** shows the results obtained for CTL and IFNγ ELISPOT analysis of four mice immunised. Two out of four mice display CTL mediated killing of Z158 and Z158m pulsed targets between 40% and 65% with no killing of the CD55 positive cell line. Interestingly, IFNγ assessment showed that all mice developed an antigen specific response in excess of 200 S.F.U. for both wild type and mutant peptides. However, at a reduced effector cell concentration of 5 x 10^5 cells only 50 S.F.U. were observed, a value being only slightly greater than control samples.

Figure 5.19: Immunisation of HHD II mice with SCR 1-3 Z158m Fc DNA

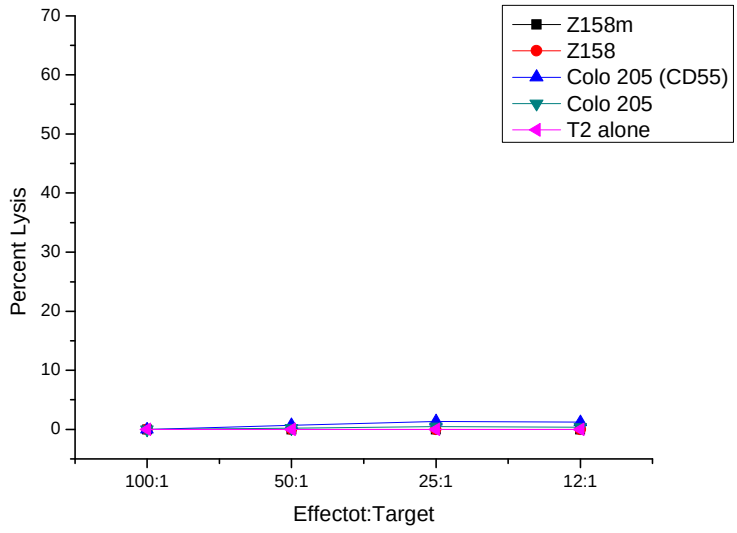
Figure 5.19 a: Cytotoxic T Cell (CTL) Assay

Standard assay protocol was followed as per **sections 2.7.1** to **2.7.4**. Target cells were labelled with 1.85MBq sodium [^{51}Cr] chromate for 1 hour with or without 100µg/ml specific peptide, washed and repeat labelled with or without specific peptide for a further hour. 5000 cells were seeded into 96 U well plates and co cultured with titrated numbers of effector cells in an assay volume of 200µl of complete media. Prior to the assay, effector cells had been co cultured *In Vitro* with 1 x 10^6 irradiated LPS blasts which had previously been incubated with 10µg/ml target peptides. Plates were incubated at 37°C 5% CO_2 for 4 hours and 50µl of assay culture media was transferred to lumaplates. Plates were allowed to air dry and were then analysed on a scintillation counter. Percent lysis was calculated relative to spontaneous and maximum lysis results.

Mouse 1 (HHDII) Immunised with SCR 1-3 Z158m Fc DNA



Mouse 2 (HHDII) Immunised with SCR 1-3 Z158m Fc DNA



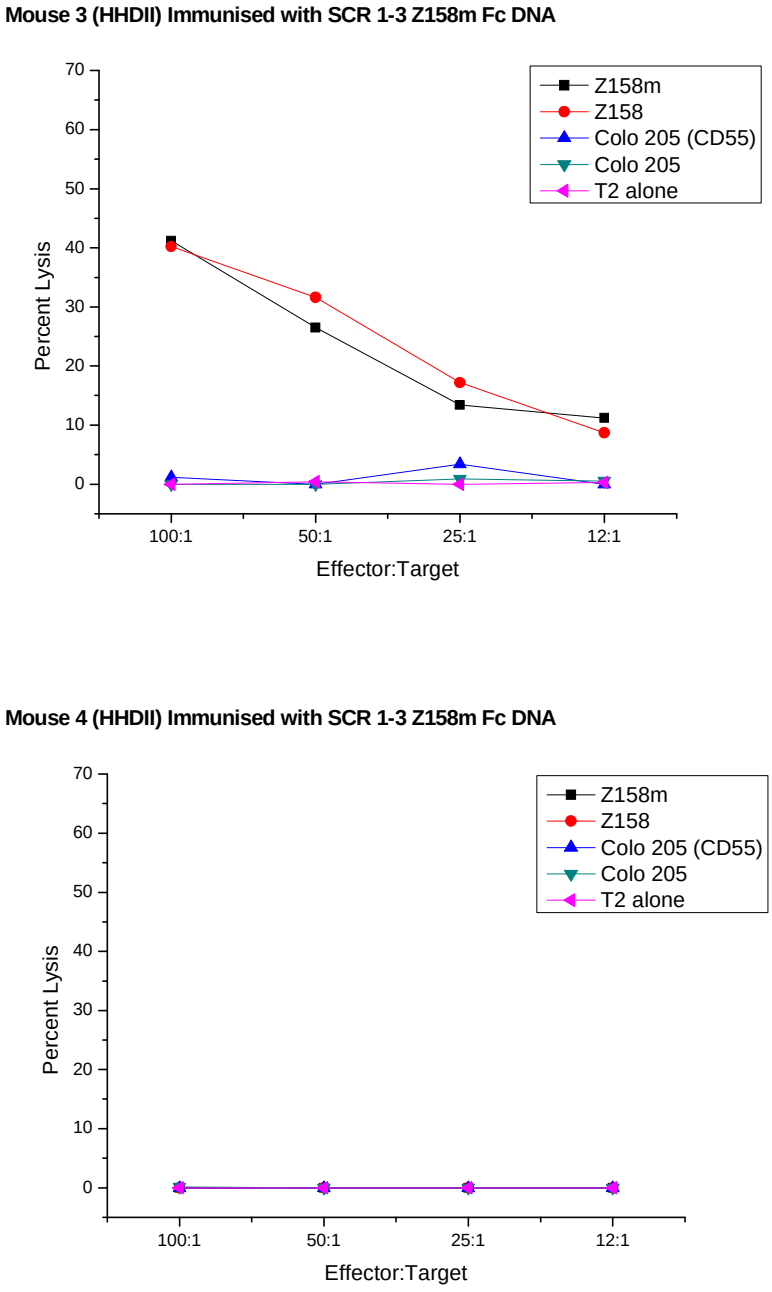
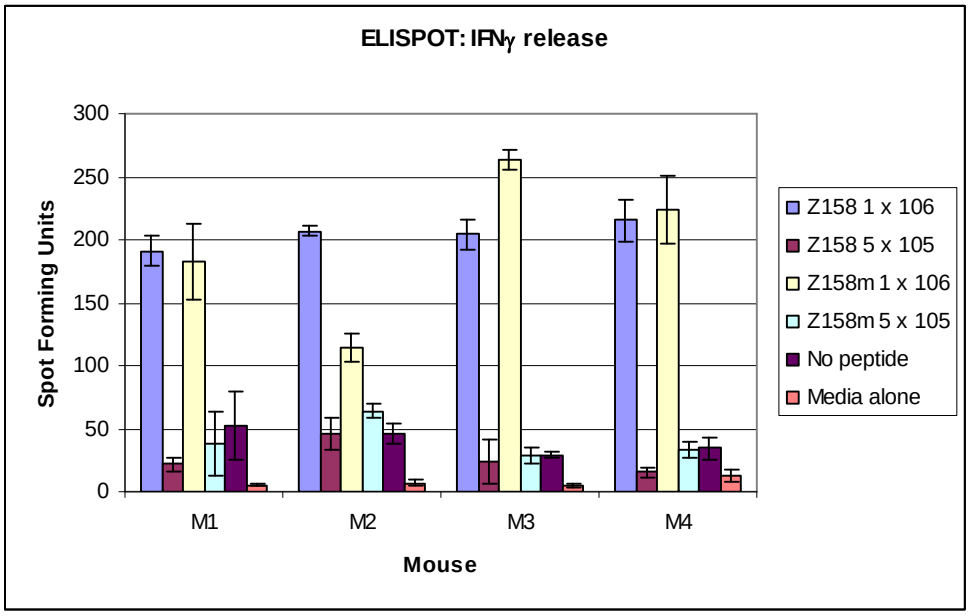


Figure 5.19b: ELISPOT analysis of Interferon γ production

Standard protocol was followed as per **section 2.7.5** utilising the ELISPOT kit from R & D Systems. 96 well assay plates were coated with cytokine specific capture antibody overnight at 4°C. Non specific binding sites were blocked and 1 x 10^6 effector cells were seeded per well. Splenocytes were then stimulated with and without 10µg/ml target peptide and control wells were set up containing media alone, cells alone and wells containing cells with PMA and Ionomycin as a positive control to test viability of cell samples. All conditions were repeated in triplicate and plates were incubated at 37°C 5% CO_2 for 16 hours. Cells were then removed and cytokine specific capture antibody was added to all wells. The development module from the kit was then used for development of the assay prior to analysis on a Bioreader 3000 Pro. Spots were counted and results expressed as the number of spot forming units [S.F.U (cells)] that produced cytokine in response to specific stimuli.



Results shown in S.F.U per 10^6 and 5 x 10^5 cells for all mice immunised from the same group.

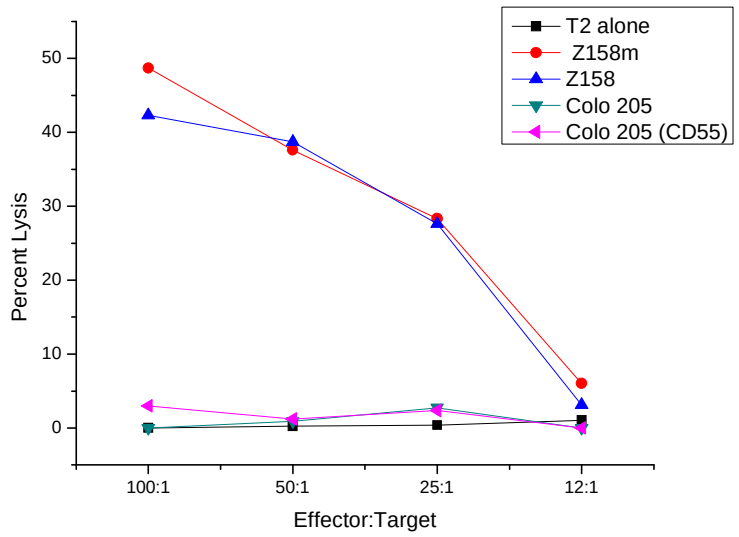
In order to assess whether an antigenic boost could be delivered in order to promote existing responses, a group of four mice were set up to be given two successive immunisations of the SCR 1-3 Z158m Fc DNA vaccine and a final I.M immunisation of 100µg SCR 1-3 Fc fusion protein in complete Freund's adjuvant. This group was assessed in parallel with a standard group of four mice immunised only with the DNA construct. **Figure 5.20** displays the results obtained for the DNA alone group. Two out of four mice generate up to 60% CTL mediated lysis of peptide pulsed T2 targets at an E: T ratio of 100:1 with no response generated towards CD55 positive cells. Two of the four mice only generated 15% CTL killing of pulsed targets with a titrating effect observed at reduced effector cell concentration. Antigen specific IFNγ release was consistent with the CTL results, with two of the four mice eliciting up to 100 S.F.U to both Z158 and Z158m peptide stimuli. **Figure 5.21** shows the results obtained for the four mice immunised twice with the DNA vaccine and once with a protein boost. As can be seen no CTL mediated killing is observed for any targets and only one mouse showed a degree of antigen specific IFNγ release to Z158 and its mutant peptide giving up to 100 S.F.U. per 10^6 cells stimulated.

Figure 5.20: Immunisation of HHD II mice with SCR 1-3 Z158m Fc DNA
Assessment of standard triple SCR 1-3 Z158m Fc DNA immunisation in direct comparison with SCR 1-3 Fc protein boost following two DNA immunisations (**Figure 5.21)**.

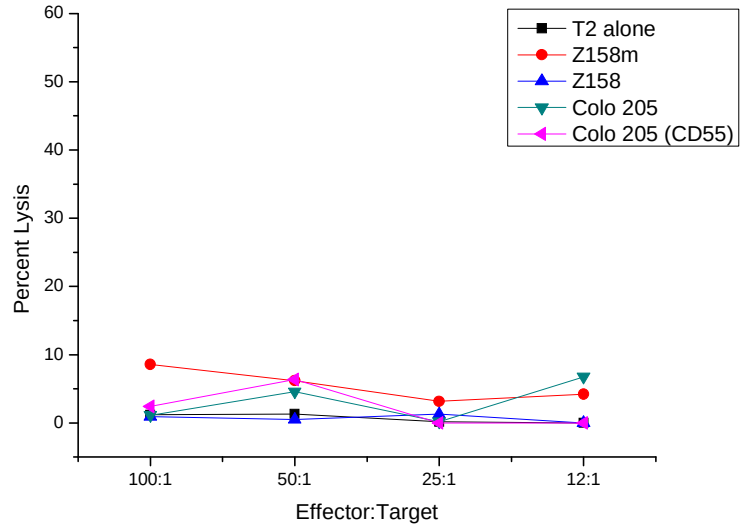
Figure 5.20a: Cytotoxic T Cell (CTL) Assay

All mice immunised three times with SCR 1-3 Z158m DNA vaccine and standard assay protocol was followed.

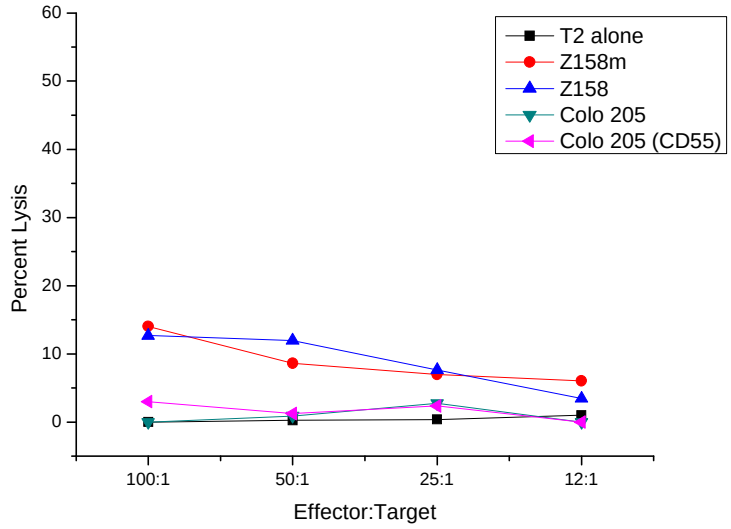
Mouse 1 (HHD II) Immunised with SCR 1-3 Z158m Fc DNA



Mouse 2 (HHD II) Immunised with SCR1-3 Z158m Fc DNA



Mouse 3 (HHD II) Immunised with SCR1-3 Z158m Fc DNA



Mouse 4 (HHD II) Immunised with SCR1-3 Z158m Fc DNA

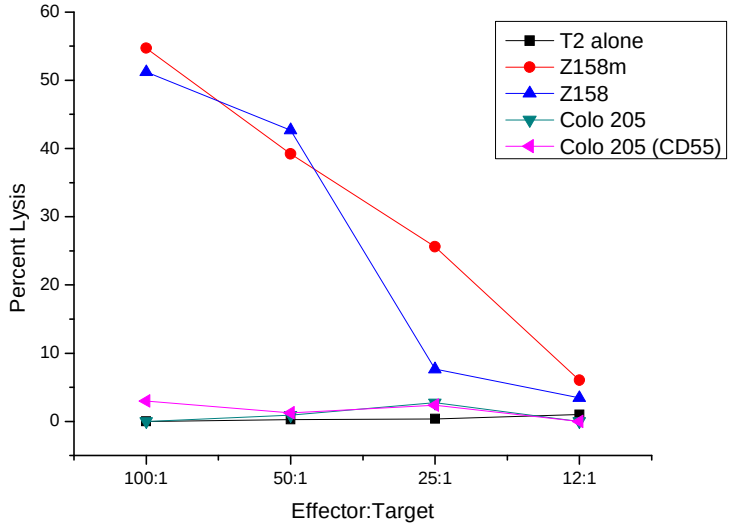
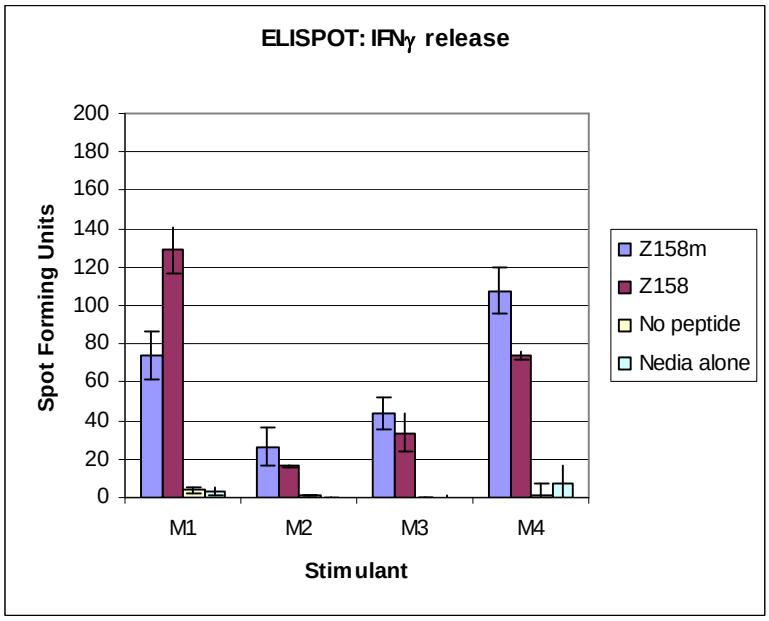


Figure 5.20b: ELISPOT analysis of Interferon γ production

Standard assay protocol was followed utilising the R & D systems kit.



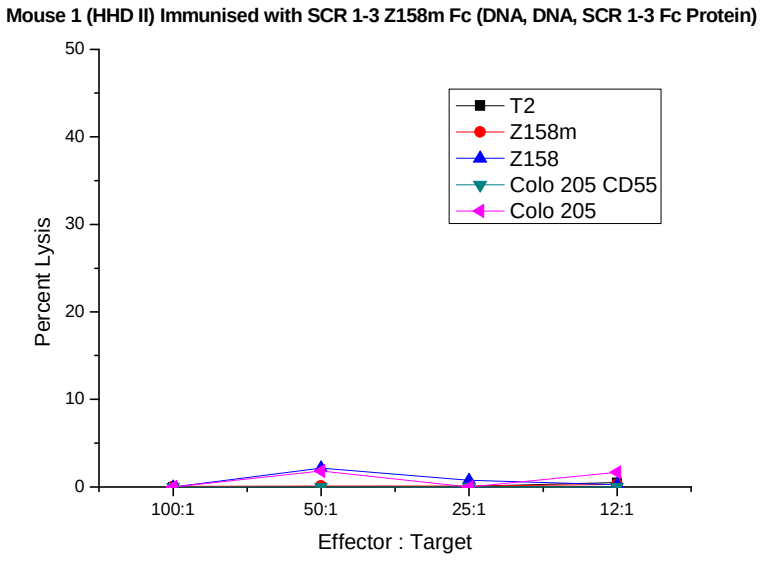
Results shown in S.F.U per 10^6 cells for all mice immunised from the same group.

Figure 5.21: Immunisation of HHD II mice twice with SCR 1-3 Z158m Fc DNA construct and once with 100µg SCR 1-3 Fc fusion protein

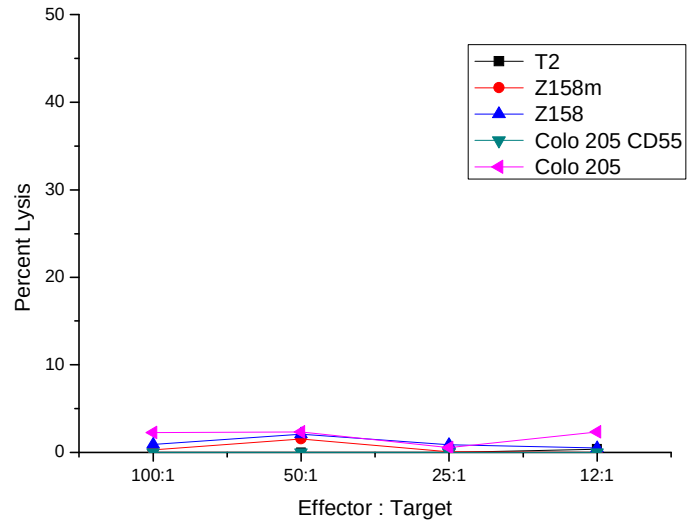
Standard immunisation protocols were followed as per **sections 2.1.3** and **2.1.6** with the protein boost being administered intramuscularly in a total volume of 100µl combined in a 1:1 ratio with incomplete Freund's adjuvant.

Figure 5.21a: Cytotoxic T Cell (CTL) Assay

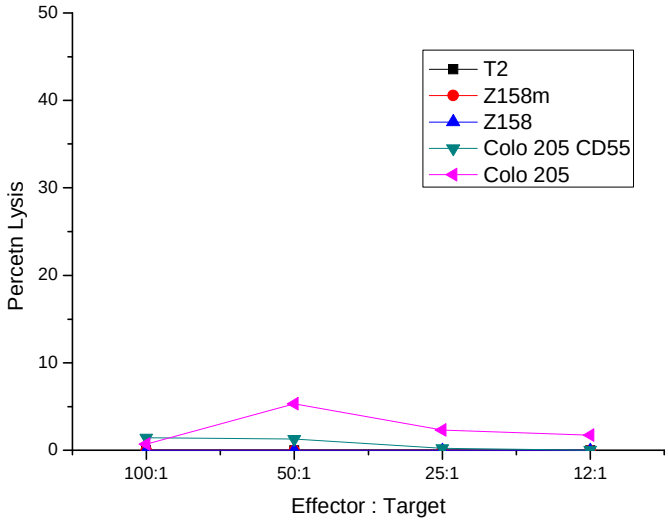
Standard assay protocol was followed.



Mouse 2 (HHD II) Immunised with SCR 1-3 Z158m Fc (DNA, DNA, SCR 1-3 Fc Protein).



Mouse 3 (HHD II) Immunised with SCR 1-3 Z158m Fc (DNA, DNA, SCR 1-3 Fc Protein)



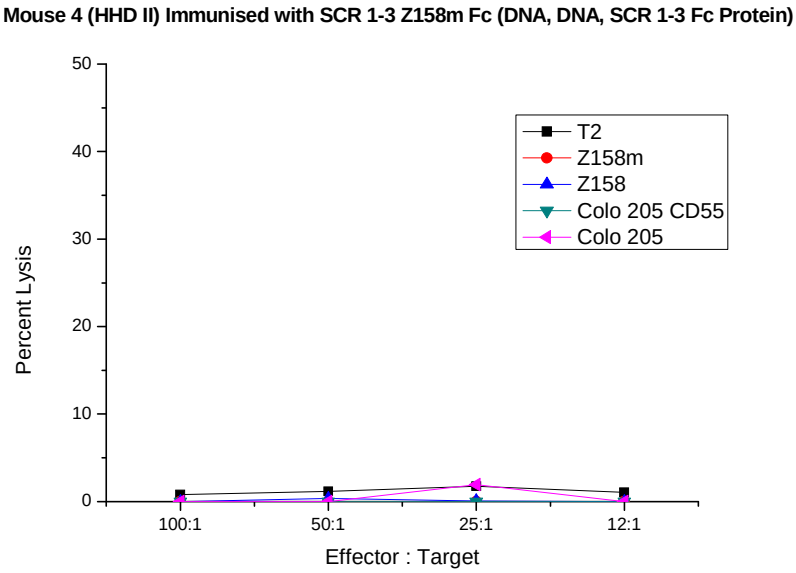
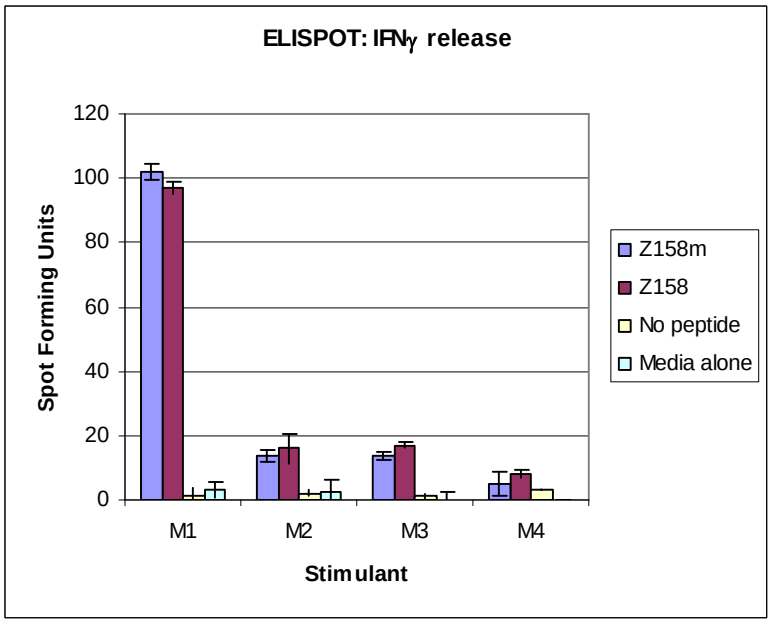


Figure 5.21b: ELISPOT analysis of Interferon γ production



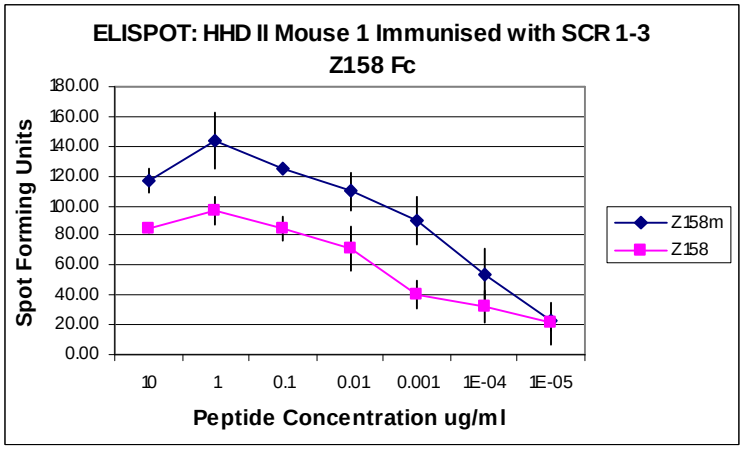
Results shown in S.F.U per 10^6 cells for all mice immunised from the same group.

A group of three mice were immunised with the SCR 1-3 Z158m Fc DNA construct in order to assess the avidity of generated responses. This was determined via antigen specific IFNγ release as assessed by ELISPOT (**Figure 5.22**). Titrations of peptides were utilised ranging from 10 to 1 x 10^{-5} ug/ml in order to generate IC_{50} values for the generated responses. All there mice elicited up to 160 S.F.U for both Z158 and the mutant peptide at a concentration of 10µg/ml in the presence of 10^6 effector cells.

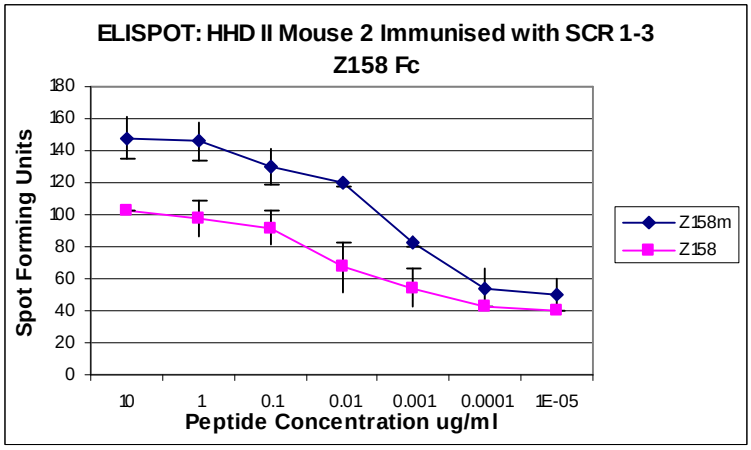
Two of the three mice showed IC_{50} values of 0.00015µg/ml and 0.0015µg/ml for Z158m and Z158 respectively. These values indicate a potential log difference in avidity of response towards the mutant peptide sequence. Mouse 2 displayed reduced values indicating a ½ log difference in avidity.

Figure 5.22: Immunisation of HHD II mice with SCR 1-3 Z158m Fc DNA to determine avidity of generated responses

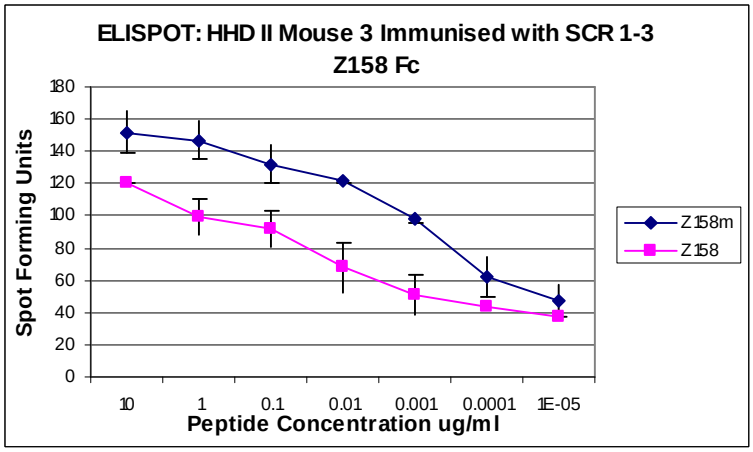
ELISPOT analysis of IFNγ production
Standard protocol was followed seeding 1 x 10^6 effector cells with titrated concentrations of Z158 and Z158m peptides.



IC_{50} Z158m = 0.00015µg/ml IC_{50} Z158 = 0.0015
~ 1 Log difference between wild type and mutated peptide



IC_{50} Z158m = 0.00015µg/ml IC_{50} Z158 = 0.001
~ 1/2 Log difference between wild type and mutated peptide



IC_{50} Z158m = 0.00015µg/ml IC_{50} Z158 = 0.0015
~ 1 Log difference between wild type and mutated peptide

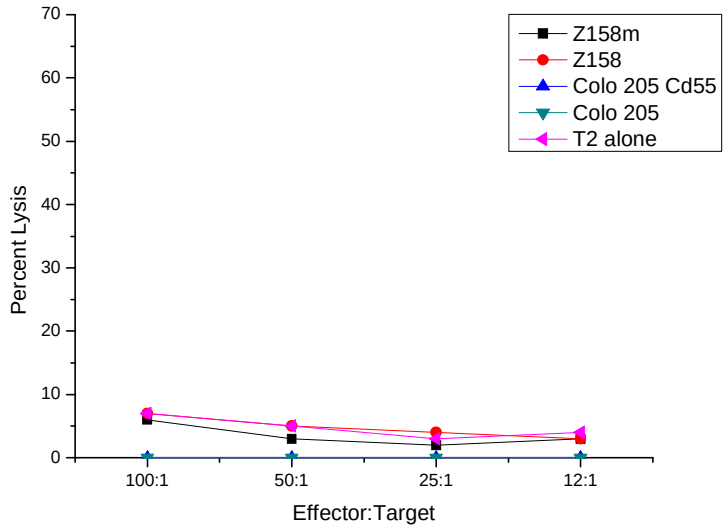
In order to confirm the need for the incorporated mutation within the DNA construct, an experiment was set up comparing a wild type immunisation of SCR 1-3 Fc with a group immunisation of SCR 1-3 Z158m Fc, both groups containing 3 mice. **Figures 5.23a/b** show the CTL results obtained from this vaccination protocol. As can be seen, no CTL mediated killing was observed for mice immunised with the wild type SCR 1-3 Fc construct in comparison to the mutant construct, which generated up to 50% lysis of both Z158 and Z158m pulsed T2 targets in two out of three mice. Mouse three of the mutant group still generated CTL mediated killing of peptide pulsed targets of up to 20%, with all mice generating limited responses to CD55 expressing cells.

Figure 5.23: Immunisation of HHD II mice with SCR 1-3 Z158m Fc DNA and SCR 1-3 Fc (Wild Type) DNA

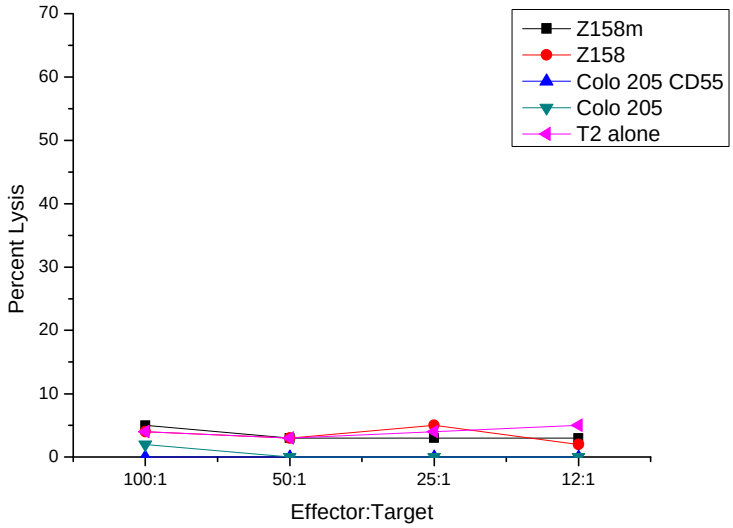
Figure 5.23a: Cytotoxic T Cell (CTL) Assay (SCR 1-3 WT Fc)

Standard assay protocol was followed as per **sections 2.7.1** to **2.7.4**. Target cells were labelled with 1.85MBq sodium [^{51}Cr] chromate for 1 hour with or without 100µg/ml specific peptide, washed and repeat labelled with or without specific peptide for a further hour. 5000 cells were seeded into 96 U well plates and co cultured with titrated numbers of effector cells in an assay volume of 200µl of complete media. Prior to the assay, effector cells had been co cultured *In Vitro* with 1 x 10^6 irradiated LPS blasts which had previously been incubated with 10µg/ml target peptides. Plates were incubated at 37°C 5% CO_2 for 4 hours and 50µl of assay culture media was transferred to lumaplates. Plates were allowed to air dry and were then analysed on a scintillation counter. Percent lysis was calculated relative to spontaneous and maximum lysis results.

Mouse 1 (HHDII) Immunised with SCR 1-3 (WT) Fc DNA



Mouse 2 (HHDII) Immunised with SCR 1-3 (WT) Fc DNA



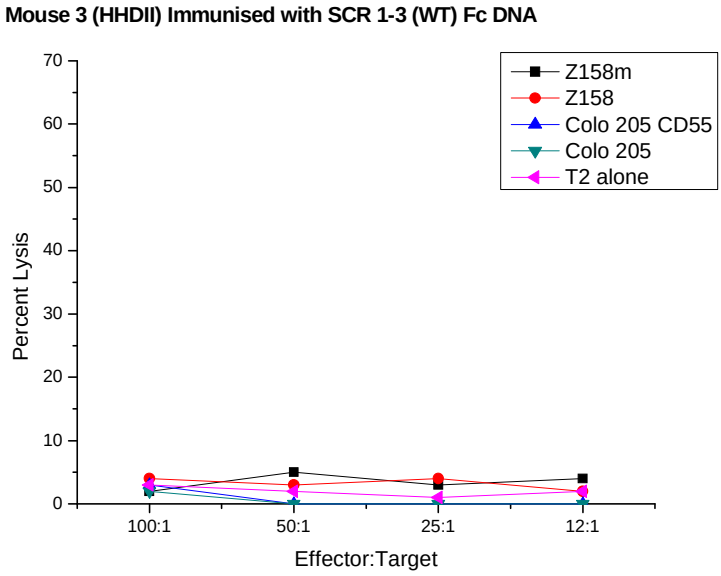
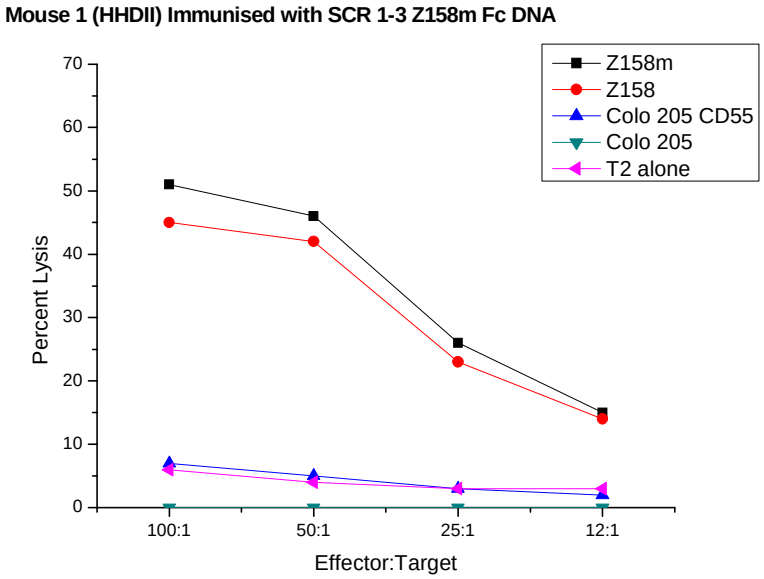
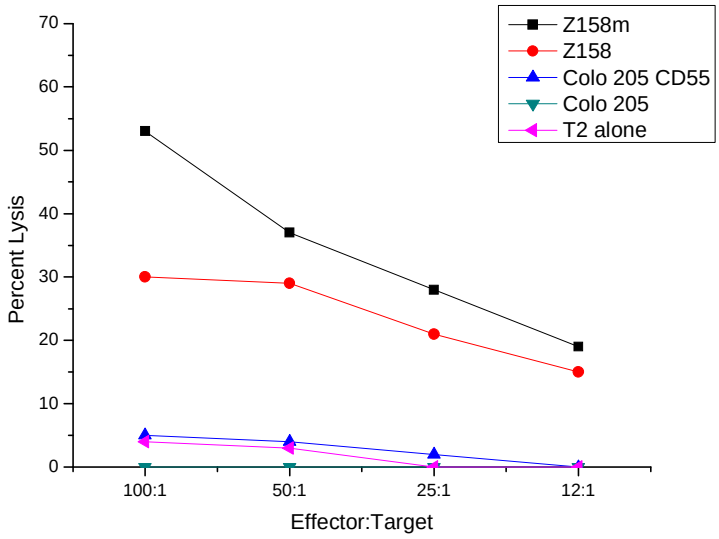


Figure 5.23b: Cytotoxic T Cell (CTL) Assay (SCR 1-3 Z158m Fc)

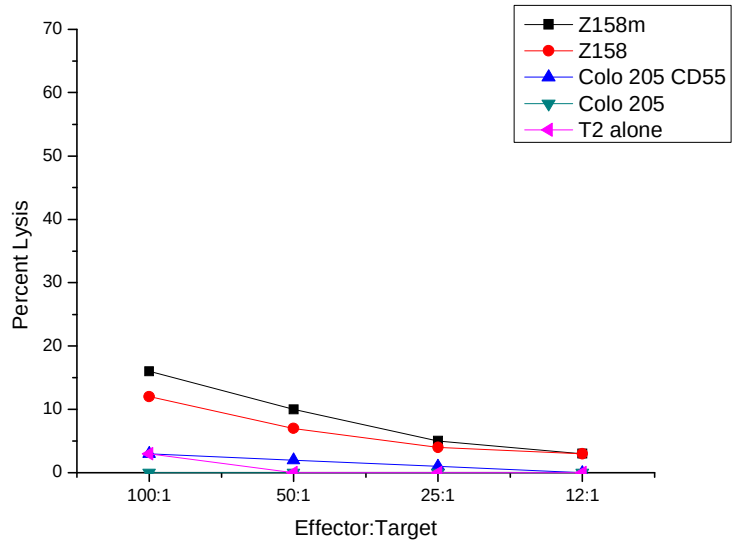
Standard assay protocol was followed as per **sections 2.7.1** to **2.7.4**. Target cells were labelled with 1.85MBq sodium [^{51}Cr] chromate for 1 hour with or without 100µg/ml specific peptide, washed and repeat labelled with or without specific peptide for a further hour. 5000 cells were seeded into 96 U well plates and co cultured with titrated numbers of effector cells in an assay volume of 200µl of complete media. Prior to the assay, effector cells had been co cultured *In Vitro* with 1 x 10^6 irradiated LPS blasts which had previously been incubated with 10µg/ml target peptides. Plates were incubated at 37°C 5% CO_2 for 4 hours and 50µl of assay culture media was transferred to lumaplates. Plates were allowed to air dry and were then analysed on a scintillation counter. Percent lysis was calculated relative to spontaneous and maximum lysis results.



Mouse 2 (HHDII) Immunised with SCR 1-3 Z158m Fc DNA



Mouse 3 (HHDII) Immunised with SCR 1-3 Z158m Fc DNA



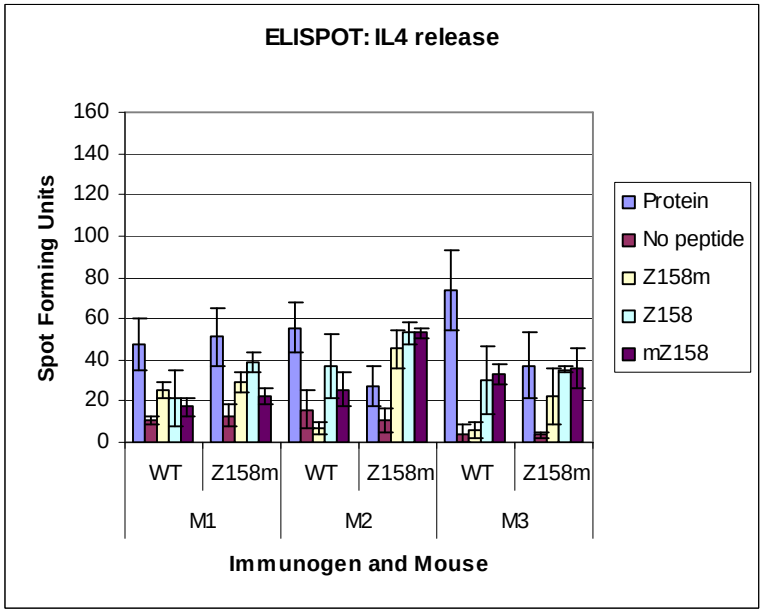
ELISPOT analysis was carried out for all mice and the murine counterpart of Z158 was synthesised and utilised in order to assess the cross reactivity for a potential self epitope. **Figure 5.23c** displays the results generated and highlights the two base differences in the murine sequence as compared to the human. SCR 1-3 Fc fusion protein was also included within the assay and both IL-4 and IFNγ release were assessed. Analysis of antigen specific IL-4 release indicated limited responses giving a maximum of 60 S.F.U. for stimulation with the complete fusion protein in mouse 3 of the wild type immunised group. Expression of IL-4 was generally consistent for all conditions although mice immunised with the modified sequence do appear to

produce slightly elevated responses. IFNγ release was consistent with previous findings with wild type immunised mice producing between 20 and 40 S.F.U for most stimuli, as opposed to Z158m immunised mice giving rise to S.F.U. in excess of 100 for both Z158 and Z158m. Interestingly two mice immunised with the mutant also indicated a response to the fusion protein as did mouse 1 of the wild type group generating approximately 80 S.F.U. Responses to murine Z158 were limited in all experimental conditions with the greatest response observed for IFNγ release from splenocytes harvested from mouse 2 of the mutant immunised group producing ~60 S.F.U.

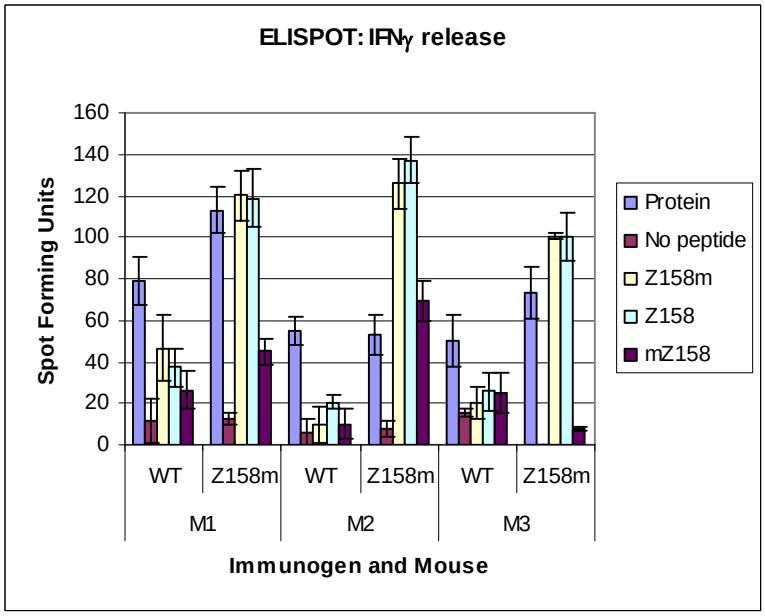
Figure 5.23c: ELISPOT analysis of IL-4 and IFNγ production

Standard ELISPOT protocol was followed assessing both IL-4 and IFNγ release from splenocytes harvested from mice immunised with wild type and modified constructs. The murine equivalent of Z158 (wild type) peptide was also synthesised and assessed within this experiment (mZ158) as a comparison for native expressed protein.

Z158:	G	L	P	P	D	V	P	N	A
Z158M:	G	L	P	P	D	V	P	N	**V**
mZ158:	G	**P**	P	P	D	**I**	P	N	A



Results shown in S.F.U per 10^6 cells



Results shown in S.F.U per 10^6 cells

5.6: Luminex analysis of splenocyte culture supernatant and mouse sera

Luminex analysis of cytokines found within the culture supernatants of *in vitro* stimulated splenocytes (used for CTL assays) and sera obtained from the cardiac blood of immunised mice was carried out to allow further elucidation of responses generated by the various construct immunisations and determination of the cytokine environment stimulated. Bioplex kits were obtained with 10plex beads enabling assessment of mouse cytokines: Il-2, IL-3, IL-4, IL-5, IL-6, IL-10, Il-12p40, IL-12p70, IFNγ and TNFα. The standard assay protocol was followed as per **section 2.5.7** using supernatant samples collected from CTL cultures (**Figure 5.23**) and sera collected from immunised mice at the time of harvest (SCR 1-3 Z158m and WT Fc DNA). Sera were also assessed which had been stored at -20°C from experiments correlating with **Figure 5.19 (SCR 1-3 Z158 Fc)** and **Figure 5.16 (SCR 1-3 Z143 Z158Fc)**.

Figure 5.24a-f display all the results obtained from one experiment analysing the concentration of ten mouse cytokines.

IL-2 analysis showed that no levels were detected greater than base line un-immunised samples.

IL-3 analysis showed that in collected sera no levels were obtained greater than base line un-immunised results. Assessment of culture supernatant however, indicated that for two mice immunised with the wild type construct and stimulated *in vitro* with fusion protein contained 37pg/ml and 17pg/ml, which appear to be significantly

greater than results from other cultures with a base line of approximately 5pg/ml found in cell cultures from un-immunised mice (**Figure 5.24a**).

Figure 5.24: Luminex analysis of supernatant from *In Vitro* stimulated splenocytes (cultured as per CTL assay) & sera collected from cardiac blood of immunised mice

The standard Bioplex protocol was followed as per **section 2.5.**7. 10plex beads were used to assess for the following mouse cytokines: IL-2, IL-3, IL-4, IL-5 IL-6 IL-10, IL-12p40, IL-12p70, IFNγ and TNFα. A 96 well plate was coated with 1.25µl of 10plex beads, washed and 25µl of samples (supernatants and sera) were added per well. The plate was incubated for 1 hour at room temperature under agitation and then washed as per standard protocol. 12.5µl of detection antibody was added per well and the plate was incubated for a further hour at room temperature. The plate was washed and 25µl of Streptavidin-PE was added per well and incubated at room temperature on a micro-plate shaker for 10 minutes. The plate was washed and beads were analysed in 125µl of assay buffer per sample. Supernatants were collected from CTL cultures used in **Figure 5.23** and sera were collected from the immunised mice at the time of cell harvest (Immunisations with SCR 1-3 Z158m and WT Fc DNA). Sera was also assessed which had been stored at -20°C from experiments correlating with **figure 5.19 (SCR 1-3 Z158 Fc)** and **figure 5.16 (SCR 1-3 Z143 Z158 Fc)**.

Figure 5.24a: IL-3 assessment within culture supernatant

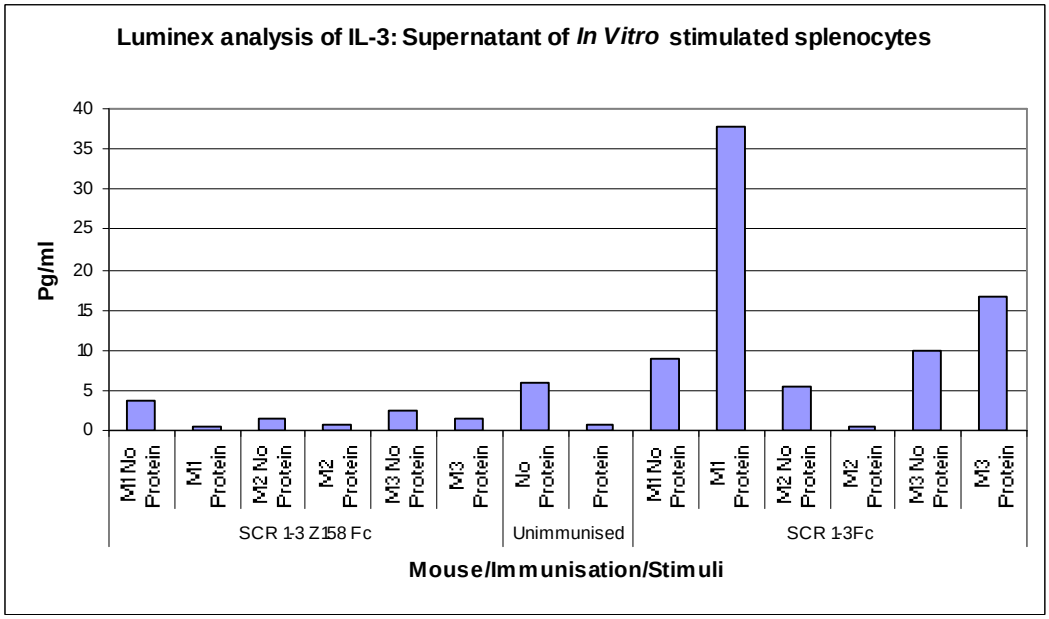


Figure 5.24a (2): IL-3 assessment of immunised sera

No results greater than un-immunised values

All assessment for both IL-4 and IL-5 in supernatants and sera samples revealed that no detectable levels were present.

Analysis of all samples for the presence of IL-6 indicated a potential Th_2 environment as supernatant samples from three mice immunised with the mutant construct contained IL-6 ranging from 400pg/ml to 700pg/ml compared to un-immunised levels of 100pg/ml (**Figure 5.24b**). Interestingly, two mice immunised with the wild type construct also show high levels of IL-6, one of 600pg/ml and one >1400pg/ml. Sera collected from these mice also showed levels of up to 70pg/ml compared to undetectable amounts present in sera from un-immunised mice. Sera from one mouse immunised with the double construct SCR 1-3 Z143 Z158 Fc also contained high levels of IL-6 of over 1000pg/ml compared with a baseline level of zero, but the greatest sera response was observed in mice immunised with SCR 1-3 Z158 Fc. Of the four mice assessed results were 800pg/ml, 2145.29pg/ml, 200pg/ml and 500pg/ml again compared to levels found in the sera of an un-immunised mouse that did not contain detectable levels.

Figure 5.24b: IL-6 assessment within culture supernatant

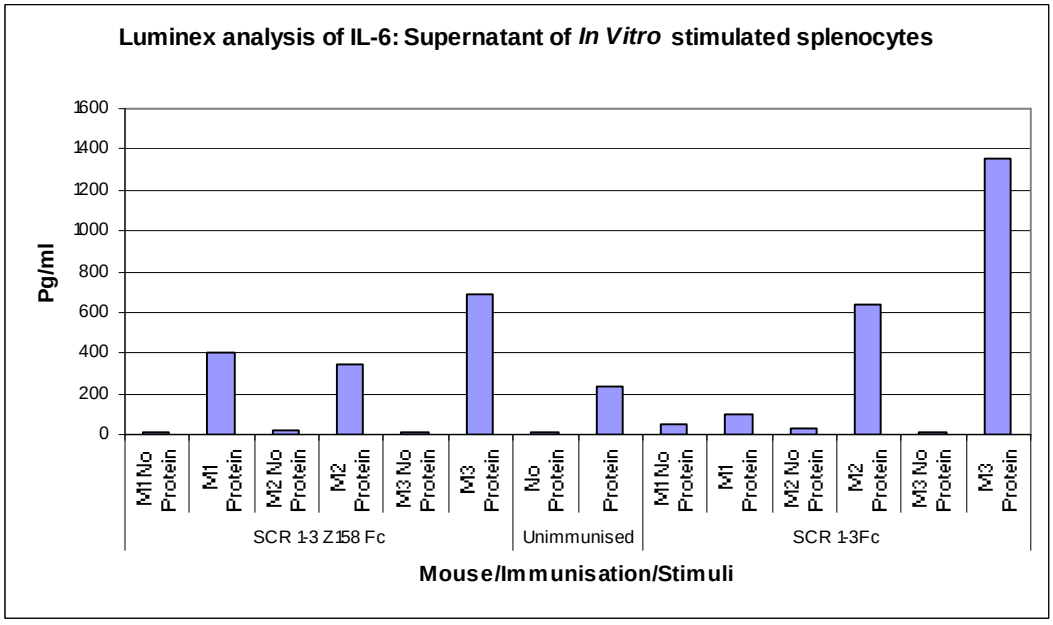
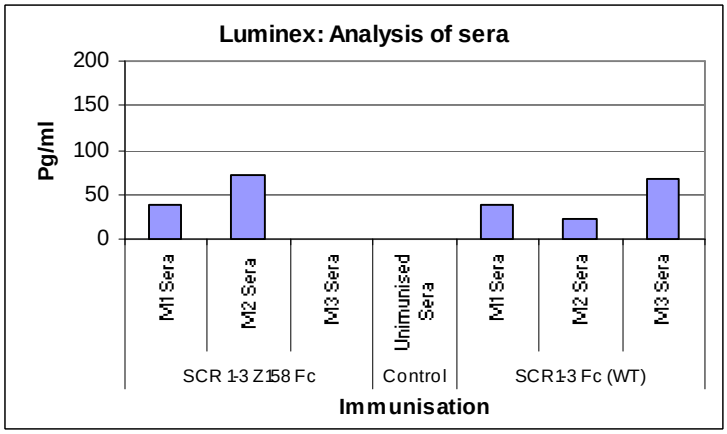
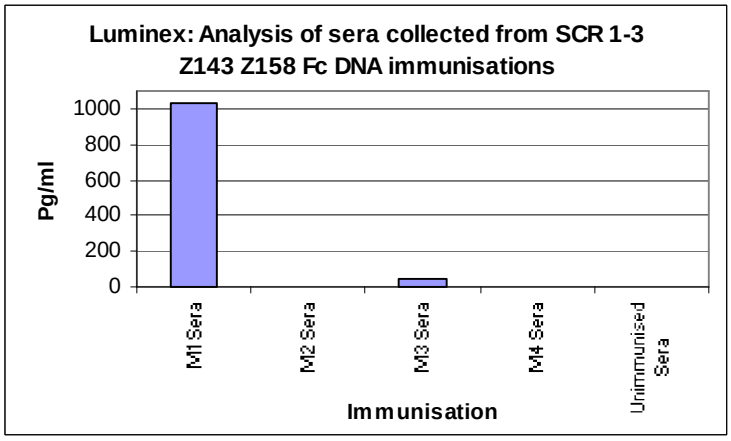
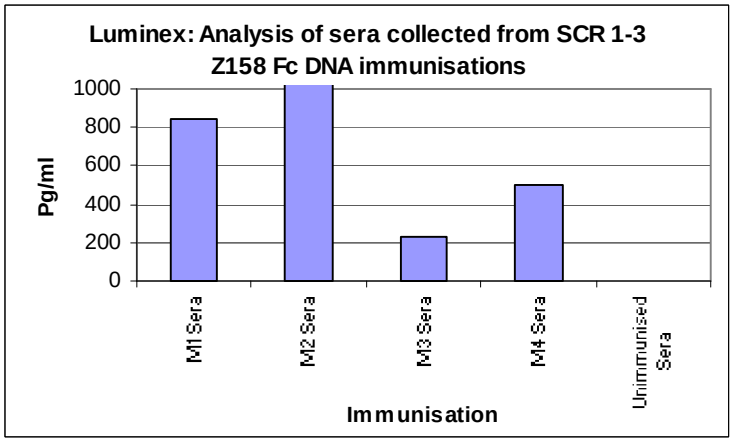


Figure 5.24b (2): IL-6 assessment of immunised sera







M2= 2145.29pg/ml

IL-10 levels were varied in the supernatant samples assessed, with almost 150pg/ml present in cultured splenocytes from an un-immunised mouse having been *in vitro* stimulated with fusion protein (**Figure 5.24c**). Supernatant from splenocytes taken from a mouse in the Z158m immunised group did contain higher levels of IL-10,

being 200pg/ml. Many samples contained lower levels upon the addition of *in vitro* applied protein, which may indicate a general dampening of a Th_1 response upon the addition of the foreign fusion protein. Il-10 was undetectable in all serum samples assessed.

Figure 5.24c: IL-10 assessment within culture supernatant

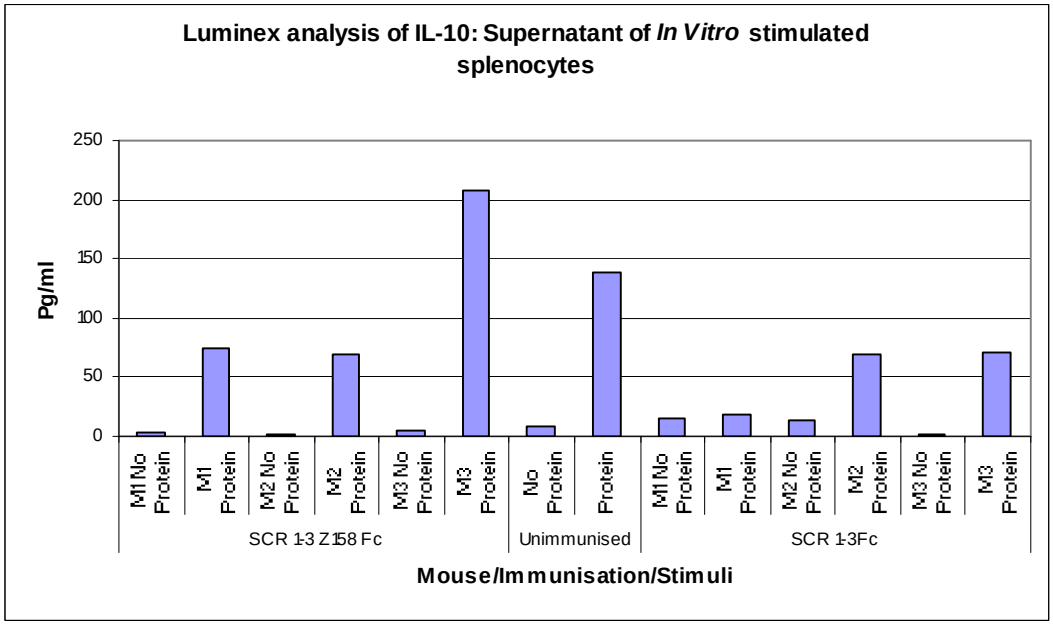


Figure 5.24c (2): Il-10 assessment of immunised sera

No results greater than un-immunised

Assessment of supernatant for the presence of IL-12p40 also showed that in all samples, a baseline concentration of ~40pg/ml was present, with cultures from only two mice from the wild type immunised group containing higher levels of >100 and >60pg/ml (**Figure 5.24d**). Assessment of mouse sera showed that one Z158m immunised mouse contained 700pg/ml and two mice from the wild type immunised group contained 650pg/ml and 500pg/ml, these values compared to an un-immunised concentration of 210pg/ml.

Figure 5.24d: IL-12p40 assessment within culture supernatant

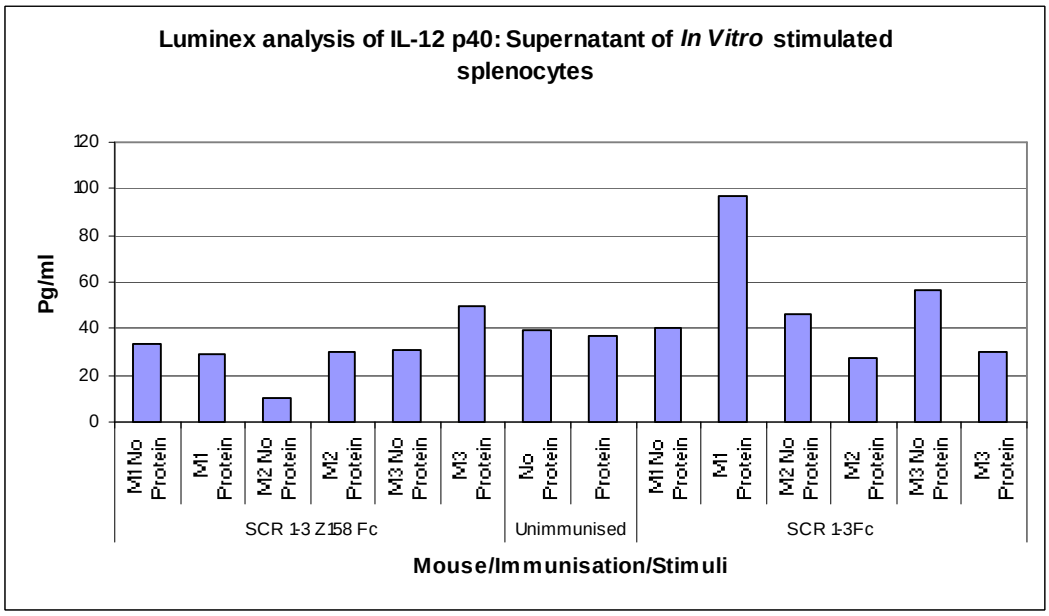
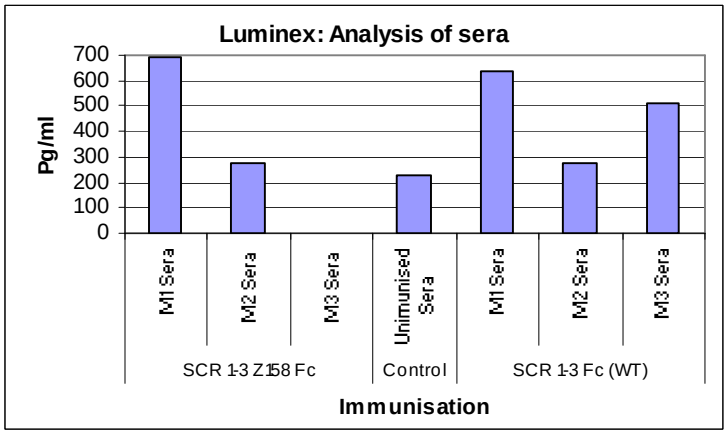


Figure 5.24d (2): IL-12p40 assessment of immunised Sera



Analysis of samples for the presence of IL-12p70 indicated that no detectable levels could be obtained.

Quantification of IFNγ levels (**Figure 5.24e**) within culture supernatants showed that only one mouse from the Z158m immunised group contained 300pg/ml and that two mice from the wild type group produced 210pg/ml and >1200pg/ml respectively. These observations were compared to baseline concentrations that were <40pg/ml in un-immunised cultures. Undetectable levels were found in the mouse serum samples.

Figure 5.24e: IFNγ assessment within culture supernatant

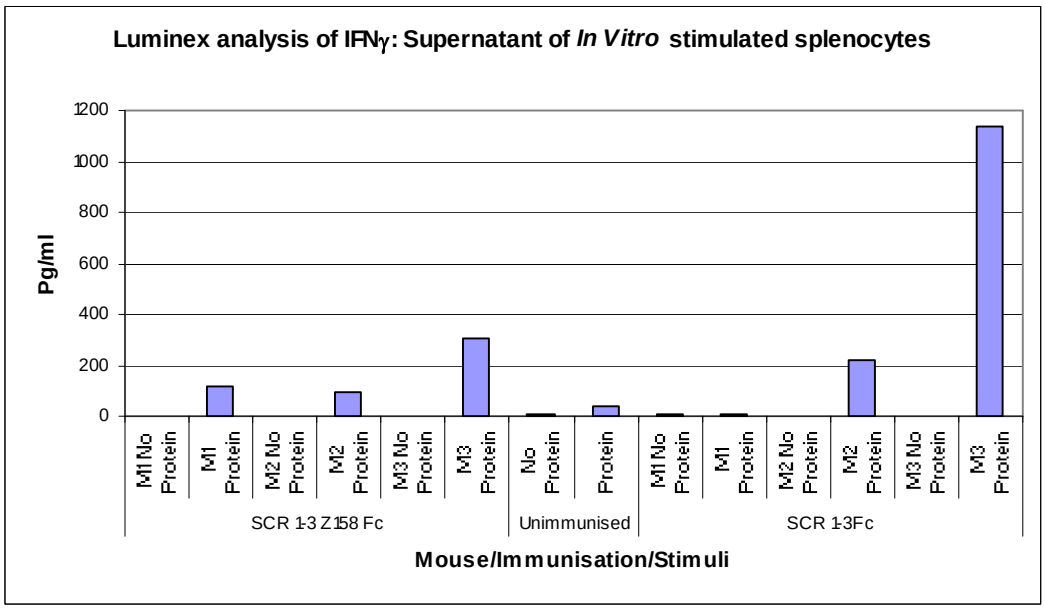


Figure 5.24e (2): IFNγ assessment of immunised Sera

No results greater than un-immunised values

Analysis of all samples for the presence TNFα generated results comparable with baseline levels.

Figure 5.24f displays summary tables combining the results obtained from the cytokine profile. The presence of each cytokine is identified by either (+) or (-) symbols that indicate the general level detected in either supernatant or serum as a direct comparison with baseline levels.

Results for antibody responses to both SCR 1-3 Fc (WT) and Z158m are shown in **Chapter 4** and are of interest when analysed with the luminex data as isotyping data would indicate the presence of a Th_2 immune environment driven by the mutant construct.

Figure 5.24f: Summary tables of cytokine profile generated by immunisation

The tables incorporate cytokine expression levels as a comparison with serum and culture supernatant form un-immunised mice. Expression is conferred by the number of (+) symbols as a relative fold increase compared with baseline concentrations. The (-) symbol denotes that no detectable levels were observed in comparison to control values.

	IL-3	IL-6	IL-10	IL-12p40	IFNγ
SCR 1-3 Z158m Fc					
Supernatants					
M1 (with)	-	+	-	-	+
M1 (without)	-	-	-	-	-
M2 (with)	-	+	-	-	+
M2 (without)	-	-	-	-	-
M3 (with)	-	++	+	+	+
M3 (without)	-	-	-	-	-
Sera					
M1	-	+	-	+++	-
M2	-	++	-	+	-
M3	-	-	-	-	-

	IL-3	IL-6	IL-10	IL-12p40	IFNγ
SCR 1-3 (WT) Fc					
Supernatants					
M1 (with)	++++	-	-	++	--
M1 (without)	+	-	-	-	-
M2 (with)	-	++	-	-	-
M2 (without)	-	-	-	-	-
M3 (with)	++	++++	-	+	-
M3 (without)	+	-	-	-	-
Sera					
M1	-	+	-	++	-
M2	-	+	-	+	+
M3	-	++	-	++	+++

Sera

	IL-6
SCR 1-3 Z143 Z158m Fc	
M1	++++
M2	-
M3	+
M4	-
SCR 1-3 Z158m Fc	
M1	++++
M2	+++++
M3	+
M4	+++

5.7: Discussion

The discovery of tumour associated antigens has enabled multiple studies to be devised analysing potential therapeutic approaches that promote anti-tumour T cell responses. However, as is well documented, the majority of currently identified antigens consist of 'self' proteins which arise due to the over expression of normal cellular genes (Rosenberg et al., 1999). Unfortunately for the battle against tumour development, the immune system has developed complex mechanisms that promote self tolerance and inhibit the potential for autoimmune disease. While the thymus induces tolerance, many autoreactive T cells escape into the periphery and still show potential for recognising tissue-specific antigens albeit at low avidity. This central tolerance is complemented by peripheral tolerance mechanisms that are mediated by the concentration of self antigens (Kurts et al., 1998 and Morgan et al., 1999). If low levels of antigen are expressed, minimal presentation occurs and self reactive "ignorant" T cells remain. However if antigens are expressed at high levels in a pro-inflammatory environment with signals inducing DC maturation, these ignorant T cells become activated. Memory T cells are also regulated by this mechanism causing a tolerant state in a cognate antigen concentration dependent manner (Kreuwel et al., 2002). These findings illustrate that induction of immune responses to overexpressed self antigens may be possible but they may be regulated by the peripheral tolerance induced by memory T cells. If low affinity T cells, which have 'escaped' into the periphery can be induced to kill cells expressing certain self antigens, this would be a potential viable methodology for successful tumour immunotherapy.

A variety of methodologies have been used to identify potential CTL epitopes for incorporation into tumour vaccines. The elution of peptides from the MHC of tumour cells has often been utilised (Maeurer et al., 1996 and Nakatsuka et al., 1999) and the results of which have been incorporated into several on line databases. Expression cloning (Aarnoudse et al., 1999, Boel et al., 1995 and Kawakami et al., 1994) has also been utilised along with the generation of overlapping peptides used to stimulate CTL responses (Beattie et al., 2004). However, these methods are labour intensive and often identify epitopes to which the immune system has either already failed to mount

a successful response or has generated regulatory T cells. A possible method for the identification of successful epitopes avoiding antigenic competition and preventing the stimulation of regulatory T cells is to utilise CTL epitopes identified from within tumour associated antigens. Many of these epitopes are shown to only bind with moderate affinity to MHC, and are thus only presented at low frequency to the T cell receptors and therefore initiate poor responses. Many studies have shown that by mutating HLA-A2 binding epitopes to preferred amino acids, the affinity of the MHC/peptide complex can be significantly increased without altering the residues bound by the T cell receptor (Ahlers et al., 2001, Irvine et al., 1999 and Sarobe et al., 1998). This process has been shown to improve the frequency and avidity of immune responses in many cases, as reviewed by Berzofsky et al (2001). It was determined to use HLA-A*201 transgenic mice as this allows epitopes to be identified that can be presented by this common human class I antigen. Specifically HHD II transgenic mice were used as several studies have shown that the absence of mouse class I molecules results in both quantitative and qualitative responses mediated by $CD8^+$ cells generated after DNA immunisation (Ramage et al., 2004 and Hüseyin et al.,2002).

HLA-A*201 epitopes were identified within the primary protein sequence of human CD55 utilising several internet based prediction algorithms. These programs incorporate data from elution studies identifying epitopes by specific residues purified from the MHC molecules of tumour cells and equate to the specificity and differential affinity with varying HLA allotypes. Varying degrees of consistency were observed across predicted epitopes as could be expected with each program possessing differing scoring systems. However several trends were noted and many of the peptides with the highest predicted binding affinities were identified by at least four of the programs used. In order to potentially overcome pre existing antigen "ignorance" to the epitopes identified, residue modifications were incorporated into the peptide sequences based on several studies (Tourdot et al., 2000, the Bioinformatics group at Leeds University and other cited groups). The premise being that by increasing the affinity for MHC, elevated levels of antigen could be presented to effector cells. The binding affinity of five of the wild type epitopes appear to be significantly enhanced by anchor modification, relative to program predicted scores, supporting previous findings. Binding potential was assessed by T2 stabilisation

assay, the results of which indicate a distinct discrepancy between predicted binding affinity and observed MHC stabilisation. Only three of the predicted wild type high affinity binding peptides displayed this property, namely Z142, Z68 and Z158, all of which generate high actual stabilisation scores with the mutants Z143, Z69 and Z158m all showing significantly increased binding affinity at both peptide concentrations assessed. While both concentrations utilised are significantly higher than amounts that would be present *in vivo*, conclusions can be drawn relative to the experimental conditions. As the T2 stabilisation assay is a viable experiment, recycling of MHC and the association and disassociation of peptide/MHC complexes must be taken into account. It is arguable that complete saturation of available MHC molecules is obtained at both 100µg/ml and 25µg/ml. However, results obtained could indicate not only the affinity of interaction but possibly the avidity relative to the number of interactions, due to the maintenance of high stabilisation scores at lower concentrations, acknowledging a decrease in score at reduced peptide presence. In comparison with all peptides assessed, Z158 and Z158m peptides produce the greatest level of observed stabilisation. The results indicate that certain anchor modifications are favourable as in the case of the Z158 to Z158m modification replacing alanine to a preferred valine residue (Falk et al., 1991). Many of the mutations assessed incorporated leucine to valine substitutions which are similar in aromatic structure and potential binding properties, which may indicate unsuccessful enhanced stabilisation for many peptides. However, this mutation was also utilised for the wild type peptides Z68 and Z142 conferring elevated scores although the wild type peptides did confer relatively high stabilisation when compared to many of the alternate sequences screened. Z69 is an example when the naturally occurring peptide contains the marginally less preferred valine as opposed to the mutated leucine residue at position 2. It is therefore, possibly expected that comparative binding properties of this wild type and mutant are similar. These results would suggest that the effectiveness of incorporated mutations largely depends upon the nature of the residue being replaced. It is clear that software based predicted binding score is by no means a definite method of ascertaining potential increased MHC affinity. It appears that there is little to no correlation between predicted values when compared with *in vitro* obtained results, confirming the necessity for T2 stabilisation assessment, a factor supported by Ramage et al., (2006) and Anderson et al., (2000) who suggest that the same computer programs did not accurately correspond with obtained

findings. A possible reason for this would be that elution studies assess individual residues as a frequency of occurrence at certain positions. While this alone may support the potential of "preferred" locations, this may not consider overall interactions within complete peptide sequences and thus structure and overall properties defined by all local interacting residues.

To determine whether a repertoire of T cells exist that recognised CD55, transgenic mice were immunised with DNA constituting either wild type constructs or constructs incorporating each of the three epitopes and compound mutants that conferred enhanced MHC binding. Of all the constructs analysed only the SCR 1-3 Z158m Fc and the compound mutant incorporating Z158m and Z143 generated antigen specific CTL mediated killing of pulsed targets, a fact supported by the expression of IFNγ in responses to antigen stimulation. The lack of responses to both the Z69 and Z143 (single modification) constructs could be due to the fact that both show reduced MHC stabilisation when compared to the Z158m peptide. Also it is possible that within the mice immunised the epitopes may not be processed and presented on the HLA-A*201. As mentioned, the Z158m peptide elicited the greatest level of MHC stabilisation and the vaccine generated up to 60 percent lysis of both the wild type and mutant epitope targets. Analysis of the avidity of responses generated indicate that a log difference is generated comparing the mutant Z158m to the wild type epitope with a frequency of over 1 in 1 x 10^4 cells expressing IFNγ in an antigen dependent manner. Furthermore, the wild type vaccine failed to elicit CTLs that recognise these epitopes even though the native peptide displayed significantly enhanced stabilisation of HLA-A*201. This implies that the epitope with higher binding affinity would have a greater occupancy upon MHC and therefore could stimulate naïve T cells. These findings agree with other studies stating that the concentration of self epitopes presented is important in breaking tolerance to self antigens (Kurtset al., 1999, Morgan et al., 1999 and Ramage et al., 2004). With respect to the current study the idea of stimulating naïve T cell responses with modified epitopes generating immune responses to wild type epitopes can be clearly observed. However, when considering the possibility of overcoming tolerance to self antigens this study can not be conclusive. Human CD55 shares 49.2 percent homology with its mouse counterpart and therefore the question of this system analysing a foreign epitope is valid. A

summary of the epitopes assessed is shown below displaying homology between mouse and human epitopes with highlighted amino acids indicating differing residues.

	Z68	Z158	Z142
Human	LVLLCLPAV	GLPPDVPNA	SLSPKLTCL
Mouse	SLLLLSTPV	GPPPDIPNA	PLPGKATCL
	33.3% homology	77.8% homology	55.55% homology

Interestingly, the epitope which generated the greatest overall responses also shows the greatest level of homology between the murine and human sequence. However when the construct was assessed via ELIPOT analysis, limited responses were observed towards the murine peptide, which would indicate that a break in tolerance cannot be implied and that further assessment of this system is required. Ideally, HHD II transgenic mice expressing human CD55 would be the most ideal model to asses this system in terms of assessing overcoming tolerance. CTLs recognising epitopes are only of true relevance if they successfully recognise naturally processed antigen. For epitopes to be presented, they would have to be processed and presented as any other whole antigen, generated by the DNA construct, thus in terms of the vaccine, results indicate that the CTL epitope was naturally processed and loaded onto the class I MHC. However, CTL mediated lysis of the colorectal carcinoma cell line transduced to express human CD55 was limited with the greatest level observed from mice immunised with the double construct SCR 1-3 Z143 Z158m Fc generating up to 15 percent lysis at an effector target ratio of 100:1. This restricted level of CTL mediated killing would possibly be due to the “low” avidity of responses generated. Other possibilities would be that processing of the human CD55 protein in mice could differ to that of human responses due to variation in the proteases in the murine model, although successful targeting of peptides may dispute this. Another possibility would be that the incorporated Fc tail could affect the nature of processed products even when considering the advantages offered by Fc targeting of vaccines (You et al., 2001 and Durrant et al., 2001). Assessment of a wider panel of potential targets, including cells which naturally express both CD55 and HLA-A202, would be needed in order to obtain more conclusive results. This double construct also appears to generate the greatest overall level of responses with respect to CTL lysis of targets

expressing both the Z158m and Z158 peptides, eliciting CTL mediated lysis of between 50 and 90 percent and also generated IFNγ at a frequency in excess of 2 cells in 1 x 10^4 seeded effectors. This observation, in combination with the fact that the construct containing Z143 alone failed to generate any significant responses could suggest the potential that the Z143 mutation has incorporated a $CD4^+$ epitope and therefore generates T cell help. This hypothesis would need further analysis incorporating the depletion of $CD4^+$ and $CD8^+$ cells. However, antigen specific release of IL-4 did not appear to be significant although possibly raised in comparison to levels observed in single mutant constructs. Another possible explanation for the responses to both the Z143 and Z142 peptides could be due the presence of epitope spreading. This phenomenon was initially characterised by an exacerbating factor in $CD4^+$ T cell dependent autoimmune disease and believed to occur due to presentation of antigens liberated by tissue destruction. Several groups have shown that epitope spreading can occur for class I restricted peptides during tumour rejection. Specifically, Markiewicz et al (2001) showed that immunisation against the single tumour peptide P1A (utilising the P815 tumour model), followed by rejection of $P1A^+$ tumour, subsequently yielded CTL activity and tumour protection against a $P1A^-$ tumour variant, and that CTLs developed were specific to a second defined epitope P1E. Mamouz et al., (2002) also confirmed this observation in a similar model, obtaining tumour rejection with the development of CTLs against additional P815 antigens which were not incorporated in their initial immunisation. While these examples are obtained from tumour immunisation/rejection models, it can be postulated that the Z158m double construct used in this study drives such a potent anti epitope (Z158) response that CTLs recognising the Z143 epitope could be enhanced by the pro inflammatory environment driven by activated DCs that cross reactivity is observed.

Analysis of the cytokine profile obtained from both sera and culture supernatants indicate the presence of a mixed environment displaying both Th_1 and Th_2 responses. Mice immunised with both the SCR 1-3 Z158m Fc DNA vaccine and the wild type counterpart generate levels of IFNγ and IL-12p40 within culture supernatants and cardiac sera respectively. Both of which are involved in Th_1 mediated responses with IFNγ promoting IL-12 induction of acquired immune responses interacting with

innate responses (Kaminski et al., 1996). Both mediate multiple effects driving T lymphocyte growth and development, stimulating release of other Th_1 cytokines. Interestingly, Il-12-p40 is also produced by B cells and dendritic cells which equate with the Th_2 profile which is also observed. A mixed profile is obtained for IL-10 results as the greatest level was observed in the supernatant from one of the wild type immunised mice. IL-10 while being a potent Th_2 cytokine is also known to reduce IL-6 expression and stimulate T_{reg} cells. However, IL-6 happens to be generated in both supernatant and sera of mice immunised with the Z158m and Z158 constructs. Many of the mice so immunised generate high levels of this cytokine that targets proliferating B cells, plasma cells and myeloid stem cells, promoting terminal differentiation into plasma cells stimulating antibody secretion and the induction of acute phase proteins. The results indicate that higher levels are located within the sera of immunised mice with the greatest levels found in mice immunised with the wild type construct. This is interesting, as it appears to support the findings obtained in chapter 4 suggesting that both the wild type and mutant constructs of Z158 elicit a defined antibody mediated response.

Pasare and Medzhitov (2003) postulated that the ligation of TLRs on dendritic cells was able to overcome $CD4^+$ $CD25^+$ T cell mediated suppression of immune responses. They initially identified that $CD4^+$ $CD25^+$ T_{reg} cells could effectively suppress $CD4^+$ $CD25^-$ T cell activation when freshly isolated splenic DCs were used as antigen presenting cells. They then proved that blocking of T_{reg} suppression was independent of co-stimulatory molecules. This was achieved utilising MyD88 deficient DC's as TLR4 signals through this pathway in a dependent manner in order to produce inflammatory cytokines and in an independent manner to upregulate MHC class II and co-stimulatory molecules (Kaisho, 2001). When these cells were utilised as APCs, neither treatment with LPS or CpG was capable of interfering with T_{reg} suppression, despite the normal upregulation of CD80, CD86 and CD40. In order to determine if cytokines mediate the observed suppression, conditioned media from LPS or CpG treated DCs was applied to the current assay using MyD88 DCs as APCs. The addition of the media led to complete blockade of T_{reg} mediated suppression. Having confirmed that cytokines were responsible for the observed blockade, antibodies were used to neutralise the cytokines found within the media obtained from stimulated DCs. The paper concluded that only blockade of IL-6

completely abrogated the ability of DC medium to block T_{reg} suppression. Other groups have supported these findings such as Powrie et al., (2003) who state that IL-6 is a requirement for the blockade of T_{reg} activity obtained from activated DCs although other TLR induced factors are also required. Kubo et al., (2004) generated conflicting results although they justified their observations in that their DC populations were myeloid derived and not the more heterogeneous population of splenic DCs used by Pasare and Medzhitov. These findings indicate that in the current model, while IL-6 could be acting as a Th_2 mediator, its presence could be inhibiting potential T_{reg} suppression of generated immune responses. Thus, the Fc targeting of DCs incorporated into the vaccine may lead to upregulation of DCs, increased IL-6, decreased T_{reg} activity and may indicate why responses are generated to both the Z158m and wild type constructs.

A potential criticism of luminex obtained data, would be in the case of supernatant obtained from cultured splenocytes being stimulated with whole Fc-fusion protein and not peptide, which may be driving new responses to the foreign protein. However, serum cytokine levels also support many of the findings although repeat assessment could possibly answer some of the questions raised.

The results obtained indicate that there is a repertoire of T cells in HLA-A*201 HHD II transgenic mice that recognise and lyse cells loaded with associated antigen. The system utilised supports current evidence for epitope anchor modification while confirming the requirement for T2 stabilisation assessment *in vitro* in combination with program based prediction protocols.

Chapter 6: General Discussion

The premise of this project was to develop potential immunotherapeutic strategies for targeting the complement regulatory protein (CRP) CD55 in the context of it being a tumour associated antigen. The cytoprotective role of CD55 and other CRPs has made them viable targets in many clinical situations, and in recent years they have become the target of many tumour antibodies. These clinical antibodies not only inhibit complement regulatory activity, enabling complement dependent cytotoxicity, but also have the potential of activating complement and associated cellular mechanisms. Antibody dependent cellular cytotoxicity relies upon the recognition of the Fc domain of antibodies by Fcγ receptors expressed by NK cells, monocytes, macrophages and granulocytes. Binding of this receptor results in the activation of phagocytic/lytic functions of these effector cells. The differential expression of CRPs on tumours has been shown to vary, with a trend towards increased levels upon many tumour types as reviewed by Fischelson et al., 2003. CD55 expression has been assessed on numerous tumours indicating significant increases upon many types studied, including colorectal and gastric cancer (Li et al., 2001). Studies utilising tissue micro arrays have assessed whether altered expression levels of CRPs can be used as a prognostic factor. Colorectal carcinoma for example, has shown to significantly overexpress CD55 and CD59, both of which have been shown to be markers of worsening prognosis (Durrant et al., 2003, and Watson et al., 2005). Interestingly in both of these studies, CD46, while expressed on most tumours, displayed limited variation in its levels. These findings support the theory that CRPs are de-regulated on many tumours and loss of one often coincides with overexpression of another. The reasons for this observation remain unclear, but the potential of either immune surveillance involving complement promoting altered expression, or the de differentiation of tumours are both possibilities.

Initial immunoscintigraphy of multiple tumour types utilising the 791T/36 antibody identified the specificity gained when targeting CD55, as explant and histological analysis indicated localisation of the antibody to the stromal and cellular components of the tumours analysed (Pimm et al., 1985). During this analysis of over 100 patients

no adverse effects were observed, a finding that supports the potential use of immunotherapeutics targeting this antigen.

Soluble CD55 has been observed in a range of pathological conditions including the synovial fluid of rheumatoid arthritis (Jones et al., 2004), and in colorectal cancer sCD55 is present in stool samples (Kohno et al., 2005). Many cell lines from differing tumours have been shown to deposit CD55 into their extracellular matrix (Morgan et al., 2002), which is mediated by MMP-7, with the SCR 1-3 domains remaining intact (Liszewski et al., 1996). Previous assessment of sCD55 has shown that it preserves its capacity to inhibit both classical and alternate pathways of complement activation (Seya et al., 1987, and Moran et al., 1992), and can also incorporate into the membranes of NK cells, decreasing their ability to mediate killing via ADCC (Finberg et al., 1992).

CD55 has been shown to be a ligand of the T cell early activation marker CD97, a member of the EGF-TM7 family of receptors (Davis et al., 1998). This was identified when CD97 transfected cells were proven to interact with red blood cells, an interaction that could be prevented by the addition of antibodies to both CD55 and CD97 (Hamman et al., 1996). Work utilising CD55 knockout mice has indicated that in these models significant increases in the magnitude of immune responses were observed (Liu et al., 2005 and Heeger et al., 2005). Miwa et al., (2002) have suggested that this observation could be due to the interaction of CD55, expressed by macrophages, with CD97 on circulating T cells. Leemans et al., (2004) have also shown that CD55/CD97 interaction has a vital role in the migration of neutrophils in models of pneumonia and inflammatory bowel disease. While these models indicate a role for CD55 in both the regulation of complement and adaptive responses, differences in expression patterns in respective species must be considered. More recently, Spendlove et al., (2006) have demonstrated that sCD55 is capable of inhibiting T cell effector function, an indication reflected in the observed decrease in proliferation and IFNγ secretion in cultures of adherent monocytes and T cell clones. These findings were confirmed utilising CD55 antibody blocking studies which neutralise the sCD55 effects.

These multiple roles of both membrane and soluble CD55 raise many questions concerning the complex interactions in which this molecule is involved. In respect to the current study, CD55 may protect tumour cells from not only complement mediated lysis, but may also protects its environment by the release of CD55 onto surrounding matrices. Soluble CD55 released into the tumour environment, while remaining functionally active, may not only prevent complement deposition onto the tumour itself, but may interact directly with activated T cells inhibiting their effector function. These observations support the directing of immune therapies towards this molecule.

The human anti-idiotypic antibody 105/AD7 was isolated from a colorectal cancer patient receiving 791T/36 antibody for radio immununo-scinitgraphy of liver metastases. The anti-idiotypic antibody recognised the binding site of 791T/36 and mimics the tumour associated antigen CD55 (Austin et al., 1989). Spendlove et al., (2000), showed that amino acid and structural homology were shown between 105/AD7 and CD55, specifically within the first two SCR domains of CD55. Durrant et al., (2001) utilised 105/AD7 as an immunogen in colorectal cancer patients, and showed that anti-idiotypic antibodies are good immunogens due to targeting of Fc receptors on antigen presenting cells, allowing efficient stimulation of both helper and T cell mediated responses. Results were measured by analysis of *in vitro* T cell proliferation, IFNγ secretion and redirected cytotoxicity in un-primed T cells from healthy donors. In 2000, results were published by Durrant et al., stating that 12 colorectal cancer patients received 105/AD7 therapy and that 10 of those demonstrated accumulation of $CD8^{+}RO$ cells or tumour killing. They concluded that treatment with this antibody could stimulate $CD4^{+}$ and $CD8^{+}$ responses, although continued immunisation was required to sustain memory responses. More recently, Pritchard-Jones et al., (2005), assessed this anti-idiotypic antibody in osteosarcoma patients following myelosuppressive chemotherapy. Their results indicated that 20 out of 28 patients showed significant T cell responses *in vitro* to the immunogen and that 13 out of 22 patients showed antigen specific IFNγ release. They also state that 9 out of 22 patients made antibody responses to CD55. They conclude that potentially therapeutic responses can be elicited, with regards to T helper responses, when vaccination was commenced post chemotherapy treatment, and that no clinically

significant toxicity was observed. These findings and on going clinical trials involving this CD55 mimicking antibody suggest the potential use of vaccination strategies exploiting the tumour associated antigen CD55.

As many tumour associated antigens have been identified, the development of new immunotherapies for the treatment of solid tumours has become possible. Focus has been placed on the production of cancer vaccines, largely due to the widespread success of vaccines in the prevention of viral diseases, which has enabled greater understanding of immunological processes and the theoretical framework for immunisation against cancer. Practical reasons for the development of cancer vaccines are also accepted, as they are easily administered to outpatients with limited side effects (reviewed by Rosenberg et al., 2004). Attempts to develop therapeutic cancer vaccines are based upon stimulating anti-tumour T cells capable of recognising cancer antigens (Rosenberg et al., 2001). The induction of $CD8^+$ cells possessing specific immune reactivity depends on interactions with other cell types such as $CD4^+$ cells and APCs, although the major effector in many models is the $CD8^+$ lymphocyte (Rosenberg 2004). Several immunising vectors have been tested in humans, including peptides, proteins, antigen presenting cells, DNA and recombinant viruses. The administration of peptides emulsified in adjuvant is currently the most effective means for the *in vivo* development of anti-tumour T cells in humans (Rosenberg et al., 2006). Several requirements are needed for the successful immunologic destruction of established tumours, including the generation of significant numbers of high avidity T cells capable of recognising tumour antigens that effectively traffic and infiltrate tumour stroma. These cells must in turn be activated at the tumour site, in order to generate appropriate effector mechanisms like cytokine secretion and direct cell lysis.

A concern with mouse tumour models is the rapid growth of extensively passaged tumour cells *in vivo*, and thus many vaccine models are assessed by their ability to prevent the outgrowth of the tumour itself. Often in these models, the presence of even large numbers of cells recognising tumour antigens in mice is insufficient to mediate tumour regression (Overwijk et al., 2003 and Speiser et al., 1997). Several factors critically affect the success of potential vaccines, including avidity of immune cells generated, inability of the tumour to activate quiescent lymphocytes, suppressor

influences generated by either the tumour itself or by the immune system itself and tolerance mechanisms including anergy (Marincola et al., 2000). All of these mechanisms for the inhibition of anti-tumour responses must be overcome in order to generate a successful vaccine.

Current successful strategies incorporate the use of the adoptive transfer of anti-tumour T cells mediating the rejection of large vascularised tumours in mice under conditions of host immunosuppression. B16 melanomas have been rejected in mice, post lymphodepletion, transferring anti-tumour T cells in conjunction with antigen specific vaccination and IL-2 (Overwijk et al., 2003). Recent human clinical trials have demonstrated this approach (Dudley et al., 2002). The trial enrolled patients with metastatic melanoma refractory to treatment with high dose IL-2 and chemotherapy. *In vitro* activated and expanded autologous anti-tumour lymphocytes plus IL-2 were transferred to the lymphodepleted patients, resulting in objective cancer regression in 6 of 13 patients. The transferred cells were shown to persist up to four months post transfer. Rosenberg (2004) summarises the effectiveness of cell transfer therapies, indicating that many alternate strategies, while potentially generating relatively high frequencies of antigen-reactive T cells, are often of low avidity for tumour recognition. This contrasts with cells generated *in vitro* from tumour infiltrating lymphocytes, which are selected for high avidity recognition of tumour antigens (Rosenberg et al., 1998). When these cells are transferred into lymphodepleted hosts, this has resulted in up to 75% of circulating $CD8^+$ cells possessing anti-tumour activity.

In contrast to solid tumours, lymphoid tumours often express co-stimulatory molecules, a fact that may explain clinical responses observed in B cell lymphoma trials to dendritic cell vaccines (Timmerman et al., 2002). Solid tumours do not express these molecules or produce inflammatory environments necessary to convert quiescent precursor lymphocytes required for tumour destruction (Rosenberg 2004). It can be observed that a major challenge in the development of cancer vaccines is the potential to develop long term memory responses in combination with the direct activation of these cells. Potential ways of achieving these targets is by enhancing stimulation of antigen presenting cells with adjuvants or by generating a pro

inflammatory environment at the tumour site in order to encourage homing of effector cells.

Another challenge in the development of effective anti-tumour vaccines is the presence of active suppressor mechanisms produced by both tumours and the immune system itself (Hori et al., 2003 and Shevach et al., 2001). A major contributor to suppressive effects is the presence of T_{reg}/ $CD4^{+}CD25^{+}$ cells that inhibit proliferation and effector functions of immune cells. Rosenberg states that a major advantage of adoptive transfer therapies is the ability to deplete host lymphocytes, including regulatory cells, prior to cell transfer, a common methodology being the use of chemo/radiotherapy. However complete lymphodepletion can not be utilised with vaccine strategies as host effector cells are critically required for developed responses. Several alternative strategies are being assessed for the specific removal of regulatory T lymphocytes.

Attia et al., (2005), show results from a clinical trial with metastatic melanoma patients treated with HLA-1*0201 restricted peptides from the gp100 melanoma associated antigen, in combination with anti CTLA-4 antibodies. Cytotoxic T-lymphocyte antigen-4 binds to CD80 and CD86 on APCs and acts antagonistically, preventing CD28 interaction that enhances T cell activation, proliferation and the production of IL-2 (Koulova et al., 1991). T_{reg} cells constitutively express CTLA-4 and are identified by their capacity to inhibit the proliferation of other T cells (Shevach et al., 2002). Attia used CTLA-4 blockade to prevent interaction of T_{reg} cells and shows that an overall objective response rate was observed of 13%, with tumour regression observed in lung, liver, brain, lymph nodes and subcutaneous sites. These responses were observed in the presence of auto immune toxicity and comparative dose regimes were analysed. 5% of patients clinically responding to treatment showed no toxicity, compared with 36% responses observed within patients displaying signs of auto immune disease. They concluded that CTLA-4 blockade in combination with peptide vaccination causes durable objective responses and that with aggressive medical management autoimmune toxicities could be controlled and reversed.

As mentioned previously, peptide vaccination strategies are shown to elicit strong antigen specific responses, although the need to develop *in vivo* high frequency phenotypically and functionally characteristic anti-tumour T cells is required for tumour elimination. Powell et al., (2004) utilised gp100 peptide immunogens in melanoma patients and showed that after a multiple course immunisation strategy, a phenotypic shift of native peptide specific $CD8^+$ T cells from early effector to effector memory ($CD27^-$ $CD28^-$ $CCD62L^-$ $CD45RO^{+)}$ displaying functional maturation. One year post final immunisation, circulating vaccine specific $CD8^+$ T cells persisted in patients' peripheral blood mononuclear cell populations, maintaining effector memory phenotype.

Mode and site of immunisation are also observed to affect the nature of T cell responses which may be generated by anti-tumour immunisation. Rosenberg et al., (2006) show in malignant melanoma patients that combining peptides within the same emulsion can alter reactivity when compared with peptides injected at separate sites. They postulate that observed results may be mediated by mechanisms based on the induction of localised non specific inflammation or competitive binding of peptides to MHC molecules. Patients were administered a highly immunogenic gp100 peptide and a less immunogenic tyrosinase peptide. Two trials were set up, the first using 31 patients immunised at separate sites and the second, using 33 patients, with both peptides being immunised at one injection site. Separate vaccination generated significant anti-peptide activity, as assessed by tetramer staining and ELISpot, towards the gp100 peptide, whereas the tyrosinase peptide generated limited responses. When the peptides were emulsified together and injected at the same site, gp100 specificity dropped and tyrosinase responses were enhanced.

As mentioned, tumour infiltration and the development of a pro inflammatory environment are required for successful anti-tumour immunotherapy. Lurquin et al., (2005), show that melanoma patients vaccinated with a MAGE-3 antigen develop anti-MAGE-3.A1 T cells at a frequency of 1.5×10^{-5} of $CD8^+$ T cells in invaded lymph nodes. However, they also show that anti-tumour cytotoxic T cells recognising alternate antigens were approximately 10,000 times more frequent than anti-vaccine cells within metastases, representing the majority of cells present. The group suggest that anti-vaccine CTLs are not the effectors that kill the bulk of tumour cells, but that

their interaction with the tumour itself promotes conditions enabling the stimulation of large numbers of anti-tumour CTLs that proceed to destroy the tumour. In this assessment, naïve T cells appeared to be stimulated as new anti-tumour clonotypes were generated post vaccination. These findings have also been identified in other studies and trials relating to melanoma vaccination strategies as summarised by Boon et al., (2005).

While many current studies and trials show limited clinical effectiveness, relative to tumour regression, successful approaches have been developed for mediating high avidity and frequency effectors. In order to promote clinically significant responses, multi-potent strategies need to be developed. By incorporating mechanisms for the stimulation of $CD4^+$ cells recognising MHC class II restricted antigens could sustain the activation and survival of $CD8^+$ effectors. Effective adjuvants such as toll like receptor agonists (Takeda et al., 2003) could be utilised in order to activate innate immune processes. The co-administration of homeostatic cytokines such as IL-15, IL-2 and other pro-inflammatory mediators could also be used to promote anti-tumour activity (Waldmann et al., 2001). The introduction of co-stimulatory molecules in combination with specific antigen may also be used to activate quiescent precursors in successful anti-cancer strategies (Hodge et al., 2001). The potential of selective suppression of immunosuppressive cytokines or regulatory cells also show promise in promoting many responses. The most successful clinical outcomes gained are often observed in adoptive transfer studies, despite the intensity of labour required, as the regression of large vascularised tumours has been demonstrated in both mice and humans.

In the context of the current study, both CTL and antibody mediated responses were observed, with the potential presence of DC released IL-6 which could be exploited for the down regulation of regulatory T cells. While further analysis of the precise methodology could be achieved, the principle of targeting CD55 as a tumour antigen remains viable. The administration of CD55 specific antibodies could not only be utilised to inhibit all CD55 interactions, but could also be exploited for the homing and development of ADCC mediated killing of tumour targets. Antibody mediated therapy, in combination with an Fc targeting DNA vaccine with the potential to elicit high avidity, high frequency CTL effectors, could be used to promote anti-tumour

responses. Incorporation of $CD4^+$ epitopes would also have the potential to sustain and promote responses, and cytokines such as IL-2 could be assessed in the promotion of effector responses observed. Initial priming of responses that could target CD55 expressing tumours would potentially disseminate cells leading to the release of alternate tumour antigens, which may promote a more significant anti-tumour response. There is also potential for the limited application of regulatory cell inhibitors such as anti CTLA-4 antibodies in order to stave off suppressive effects, enabling the generation of a more complete pro-inflammatory environment.

In conclusion, the methodology used and results obtained indicate the potential successes for this strategy. Questions are raised due to the avidity of responses generated and the functional abilities of antibodies raised. Future assessment would need to incorporate human CTL responses to the modified epitope, and immunisation strategies within transgenic mice expressing both HLA-A2 and human CD55 would be needed in order to assess the potential for overcoming immune tolerance. The inclusion of MHC class II epitopes, required for licensing of antigen presenting cells, would have the potential to increase the efficiency of responses generated. Further assessment of the importance of the Fc region of the construct could be elucidated by the addition of a 'stop' codon into the vaccine itself, and the frequency of responses determined for a more complete analysis of the current construct.

CD55 remains a viable target for the development of anti-tumour immunotherapeutics. Further characterisation of responses generated in this study, assessing the CD55 inhibitory potential of antibodies generated and the ability of $CD8^+$ cells to mediate killing of tumours expressing CD55, and the incorporation of methods to stimulate multiple arms of the host immune system should be utilised for the development of a potential therapeutic treatment.

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