

# **Targeting the hypoxic tumour phenotype with specific T cell immunotherapy**

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Trinity term 2004)

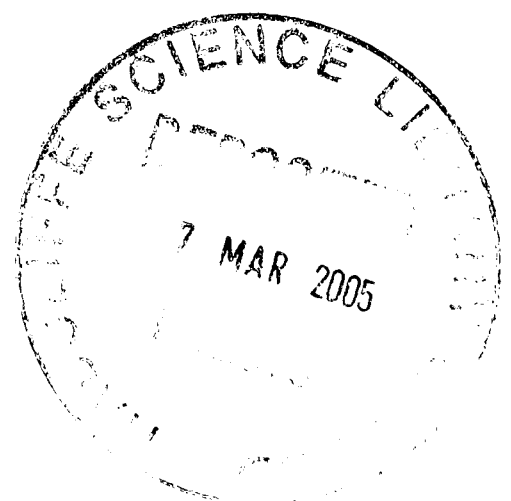
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The hypoxic tumour phenotype is a widespread phenomenon in cancers that correlates with malignant behaviour and poor clinical outcomes. The induced expression of hypoxia-regulated genes through post-translational stabilization of hypoxia-inducible factor (HIF), represent a novel class of over expressed tumour antigens that may bear relevance for specific T cell immunity. To assess whether hypoxia inducible proteins can elicit specific T cell responses in vivo, experiments were carried out using complementary models. Initially, specific T cell responses to a hypoxia-regulated antigen  $\beta$  galactosidase ( $\beta$ gal) in a mouse melanoma model were investigated. The responses correlated to hypoxia-induced expression of  $\beta$ gal in vitro, and resulted in anti-tumour effects in vivo. Next, the utility of human carbonic anhydrase 9 (CA9) as a tumour antigen was explored. In particular, renal cell carcinomas (RCC) which constitutively express CA9 as a result of HIF stabilization through loss of von-Hippel Lindau (VHL) tumour suppressor gene, were targeted. Since the HLA-A2 restricted peptide CA9<sub>254-262</sub> was reported to be immunogenic and endogenously presented in human cells, peptide specific T cell responses were induced in vivo using HLA-A2 transgenic mice, and monitored with novel CA9-specific tetramers. Subsequently, CA9 specific T cell responses in HLA-A2 healthy donors and RCC patients were generated in vitro which recognized exogenous but not endogenously presented peptide as a result of low avidity interactions, or failure of correct processing and presentation by antigen presenting cells.

To assess whether anti-angiogenic therapy can be combined with vaccination strategies, the combination of anti-angiogenic and specific T cell immunotherapy was explored using a novel 'metronomic dosing' regime of cytotoxic agent cyclophosphamide. A synergistic anti-tumour effect in a murine melanoma model was demonstrated, while sparing a population of memory T cells that were subsequently capable of re-expansion.

Finally, a novel iron chelator desferri-exochelin was found to induce HIF and downstream targets, suggesting that specific T cell immunotherapy against hypoxia-regulated targets may be enhanced through chemical means. Additionally, desferri-exochelin was anti-angiogenic in vitro, and inhibited tumour cell proliferation through HIF independent pathways.

## **Declaration of DPhil thesis authorship**

I declare that the thesis submitted entitled “ **Targeting the hypoxic tumour phenotype with specific T cell immunotherapy**” represents work performed entirely by myself with the following exceptions:

- (chapter 3) The stably transfected tumour cell line Z16 was a gift from the Prof Ian Stratford, Department of Pharmacy, University of Manchester; as were immunohistochemistry staining of Z16 tumour sections which were performed in collaboration.
- (chapter 4) All peptides in this and the following chapter were obtained commercially or manufactured within the Human Immunology Unit, WIMM, Oxford. A2 and A2K<sup>b</sup> MHC I transformed bacteria were generated within the group by Ms Dawn Sheppard and Dr Edward Choi
- (chapter 5) All blood specimens (renal cancer patients) were provided by Dr David Cranston, Department of Urology, Churchill Hospital; and Dr Andy Protheroe, Department of Medical Oncology, Churchill Hospital. Immunohistochemistry staining of human renal tumour sections was done in collaboration with the Dr David Davies, Department of Histopathology, John Radcliffe Hospital, Oxford. Human renal tumour line RCC4 were provided by the research services, Cancer Research UK. Control Melan-A tetramers were provided by Dr Mariolina Salio and Joelle Renneson.
- (chapter 6) This published work (Can Res. vol 63: 8408, 2003) was declared a joint authorship with Dr Ian F Hermans, who performed the majority of the in vivo work. The recombinant plasmid DNA-mel3 and virus Vacc-mel3, and some tetramers used in this paper were provided by Dr Michael J Palmowski.
- (chapter 7) This published work (Can Res. vol 62: 6924, 2002) was based on the drug desferri-exochelin supplied by Dr Lawrence D Horwitz, University of Colorado, Denver CO, USA; who has declared a financial interest in the company Keystone Biomedical Inc. which produced the drug. In addition, C4.5 and mutant Ka13 cell lines, and recombinant plasmids pGL3PGKX3 and pcDNA1/Neo/HIF-1 $\alpha$  were a gift from Dr Peter Ratcliffe, Wellcome Trust, Oxford.

I declare that I have not submitted this work for any other degree.

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## Chapter 1. Introduction

### 1.1 The immunological basis of cancer

It was a Surgeon, Dr William Coley, at the Memorial Sloan-Kettering Cancer Centre in New York over a century ago who first ignited the hope of targeting cancer with immunotherapy, with the seminal observation that spontaneous anti-tumour responses in some patients followed on from preceding infectious episodes. The team subsequently met with limited clinical success with injections of extracts of pyogenic bacteria, better known as ‘Coley’s toxins’ into patients in the hope of effecting tumour regression. Following this, *in vivo* work in syngeneic mice with transplantable carcinogen-induced tumours demonstrated rejection of putative antigens that were tumour specific (Baldwin, 1955; Klein et al., 1960; Prehn and Main, 1957). The landmark concept of ‘immune-surveillance of cancer’ put forward by Thomas in 1959 and then Burnet in 1970 (Burnet, 1970; Thomas, 1959) hypothesised that the immune system actively recognises and eliminates tumours that arise spontaneously based on the expression of tumour-associated antigens (TAA). This initial excitement of the prospect of cancer-directed immunotherapy gradually gave way to scepticism and disillusionment when it emerged that transplantable carcinogen-induced tumours were not the same as spontaneous tumours in that the latter were largely non-immunogenic (Hewitt et al., 1976). Experiments comparing spontaneous and chemically-induced cancer incidences in athymic nude mice (immunocompromised mice with absent T cell development) versus wild-type mice did not reveal any differences (Rygaard and Povlsen, 1974; Stutman, 1979), as did studies of cancer incidences in immunocompromised patients (Gatti and

Good, 1971; Penn, 1988), with the notable exception of virus-associated tumours. These particular cancers showed increased frequencies in such patients, and were all linked to virus infections; lymphomas with the Epstein-Barr virus (List et al., 1987), Kaposi's sarcoma with human Herpesvirus 8 (Mesri et al., 1996), cervical cancer with human Papillomavirus (Boshart et al., 1984). Thus, the evidence at the time suggested that although immune-surveillance did afford some protection against virus-associated tumours by either preventing or controlling infection, the failure to observe any differences in non-pathogen related tumours was taken as a strong argument against it.

On hindsight, those initial observations comparing cancer incidences in nude mice with wild-type were flawed in several aspects. It is now clear that nude mice are not completely immune incompetent, and have detectable populations of  $\alpha\beta$  T cell receptor-bearing lymphocytes (Ikehara et al., 1984). Also, there exists other non-thymic dependant lymphocytes such as natural killer (NK) cells and some  $\gamma\delta$  T cell subsets (Hayday, 2000). The experiments did not take into account the variable susceptibility of different mice strains to methylcholanthrene (MCA) induced tumour formation (Heidelberger, 1975). Lastly, the studies by Rygaard *et al* (Rygaard and Povlsen, 1974) spanned a relatively short period of 3-7 months, which may not have allowed for the development of spontaneous tumours.

A renaissance for the immune-surveillance hypothesis emerged between 1994 and 1998, when several key experiments established the importance of interferon-gamma ( $\text{IFN}\gamma$ ) and perforin in protecting against spontaneous and MCA induced tumours in mice.

Fibrosarcomas grew faster in mice treated with neutralising antibodies to IFN $\gamma$ , and over-expression of a dominant negative mutant of IFN $\gamma$  receptor in tumours enhanced their tumorigenicity when transplanted into naïve hosts (Dighe et al., 1994). Mice with defects in IFN $\gamma$  signalling via signal transducer and activator of transcription (STAT1) also showed increased sensitivity to the tumour-inducing capacity of MCA (Kaplan et al., 1998). There was an increased incidence of spontaneous tumours in IFN $\gamma$  <sup>(-/-)</sup> and perforin <sup>(-/-)</sup> mice (Street et al., 2001), and also a higher incidence of spontaneous disseminated lymphomas in perforin <sup>(-/-)</sup> mice with concomitant p53 <sup>(-/-)</sup> (Smyth et al., 2000b). More definitive data supporting the cancer immune-surveillance process came through the use of transgenic mice lacking the recombination activating gene (RAG) 1 or 2, which only possessed NK cell mediated immunity. Shankaran *et al* (Shankaran et al., 2001) reported on the importance of T and B lymphocytes in protection against chemically induced sarcomas and spontaneous epithelial tumours in these mice. In fact, many additional studies involving various individual components of the immune response supported the role of both innate and acquired immunity in cancer immune-surveillance (reviewed by Dunn *et al* (Dunn et al., 2002). Interestingly, Qin and Blankenstein (Qin and Blankenstein, 2004) recently posited an alternative view, suggesting that the anti-tumour effects of IFN $\gamma$  on MCA induced tumours were related to a non-immune tissue repair response involving encapsulation of MCA. Also, they suggested that the increased incidence of spontaneous tumours in aged, life-long immuno-suppressed mice could partly be explained by increased opportunistic infections and ensuing chronic inflammation, which has a known association with tumour development.

The evidence from human studies was seemingly in favour of the immune-surveillance hypothesis. It was clear that some of the higher cancer risks in transplant and primary immuno-deficiency patients were associated with increased susceptibility to tumour-associated viruses (*vida supra*). However, further data from transplant patients on immuno-suppression showed increased incidences of non-virus associated tumours (Birkeland et al., 1995; Penn, 1995; Pham et al., 1995; Sheil, 1986). In addition, data demonstrating positive correlation between the presence of tumour infiltrating lymphocytes (TIL) and patient survival indirectly implicated the relevance of immune-surveillance (Clemente et al., 1996; Lipponen et al., 1992; Naito et al., 1998). Finally, a more recent refinement of the immune-surveillance hypothesis has been proposed by Dunn *et al* (Dunn et al., 2002) . More than just simply eliminating nascent tumour cells, the immune response also shapes those tumours in a Darwinian fashion into more immune-resistant forms, thereby selecting ultimately for tumours that are less immunogenic. This has been supported by studies in mice involving re-passage of transplantable tumours through immuno-competent hosts, and generation of tumours variants with reduced immunogenicity (Urban et al., 1982; Uyttenhove et al., 1980). This process of immuno-editing of tumours is based on their inherent genetic instability, allowing for mutations and modification of expressions of genes involved in such key processes as tumour antigen processing, presentation via the major histocompatibility complex (MHC) and IFN $\gamma$  signalling pathways.



## **1.2 Effector mechanisms of anti-tumour immunity**

The immune responses to tumours and their antigens comprise innate as well as adaptive responses.

### **1.2.1 Natural killer (NK) cells**

Being a part of the innate immune response, NK cells were originally identified on a functional basis because of their ability to lyse certain tumours in the absence of previous stimulation. They represent a lymphoid population that, in contrast to T or B lymphocytes, does not express clonally distributed receptors for antigen. It was the seminal idea by Klaus Karre in 1981, that proposed that NK cells operated by detecting information that was missing in the target and present in the host; the ‘missing self’ hypothesis (Karre, 2002). This ‘information’ has since been established as the MHC I molecule, and it is the binding of MHC I on normal cells to inhibitory receptors (KIR) on NK cells that prevents cell lysis. MHC I negative RMA lymphomas and not wild type lymphomas were shown to be rejected by NK cells (Lanier, 1998). However, tumours expressing MHC I related glycoprotein MIC A were also found to be susceptible to NK mediated killing despite the presence of conventional MHC I (Bauer et al., 1999), a phenomenon associated with MIC protein expression by the tumour interacting with NKG2D receptors on NK cells. NKG2D is now known to potentiate activation of other cell types including CD8+ T cells,  $\gamma\delta$  T cells, NKT cells and macrophages through an expanding list of ligands, and is regarded as a key signal in tumour recognition. This has been indirectly supported by the demonstration that tumours sometimes evade NK cell



killing by the shedding of soluble MIC proteins which bind to and down regulate NKG2D (Groh et al., 2002; Wu et al., 2004).

### 1.2.2 $\gamma\delta$ T cells

$\gamma\delta$  T cells are intraepithelial lymphocytes (IEL) that reside primarily in epithelial tissue, particularly in the skin and gut mucosa. Early in development, they harbour an enormous potential for TCR diversity despite a restricted variable gene segment repertoire. After birth, there is a progression to oligoclonality through peripheral antigen mediated selection, until a single subset (V $\gamma$ 9-V $\delta$ 2) of  $\gamma\delta$  T cells predominates. Two groups (Diefenbach and Raulet, 2001; Girardi et al., 2001) have shown that  $\gamma\delta$  T cells are crucial for immune surveillance against malignant epithelial tumours. They have reported that both epidermal and intestinal  $\gamma\delta$  IEL express the NKG2D receptor, and are activated by NKG2D ligands Rae-1 and H60 in mice (Diefenbach et al., 2001), or MIC A and B in humans (Groh et al., 1999). These inducible ligands represent stress responses, and from the standpoint of immune surveillance of tumour formation, may signal the effect of genotoxic damage by stresses such as UV light on the skin, and gut-derived carcinogens on the intestinal epithelia. Killing of both Rae-1 and H60 positive skin cells by  $\gamma\delta$  IEL requires both NKG2D and  $\gamma\delta$  TCR, suggesting that engagement of NKG2D provides some form of co-stimulatory signal for TCR, resulting in cytokine expression and cytotoxicity.  $\gamma\delta$  T cells thus may represent the front line of innate defence against tumours in such specialised epithelial surfaces.

### 1.2.3 Natural killer T (NKT) cells

NKT cells constitute a lymphocyte subpopulation that might have important activating and regulatory functions, on the basis of reactivity through CD1d recognition (Bendelac et al., 1995) and Th1/ 2 cytokine (IFN $\gamma$  and IL4 respectively) profiles. They are phenotypically defined by the expression of NK receptors NK1.1 (CD161) and a T cell receptor (TCR). However, NK1.1+/TCR+ cells are a heterogenous group, with several categories having been described (Kronenberg and Gapin, 2002). It was the murine T cell population bearing the invariant chain V $\alpha$ 14 (V $\alpha$ 14i NKT) that was shown to be important in resistance to methycholanthrene-induced fibrosarcomas (Smyth et al., 2000a). An emerging body of literature also supported the hypothesis that treatment with the glycolipid  $\alpha$ -galactosyl ceramide ( $\alpha$ -GalCer) was beneficial in the prevention of tumour growth and metastasis (Cui et al., 1997; Smyth et al., 2002), which was mediated by the V $\alpha$ 14i NKT cell. Activation of V $\alpha$ 14i NKT by  $\alpha$ -GalCer *in vivo* has been suggested to lead to NK cell activation and IFN $\gamma$  production, through the intermediary action of DC derived IL12 (Kitamura et al., 1999; Tomura et al., 1999), and also augmentation of CD4+ and CD8+ T cell responses (Hermans et al., 2003). The broad array of potential anti-tumour responses induced by NKT cells in mice (V $\alpha$ 14i T cells) and humans (V $\alpha$ 24i T cells), make such immune-based cancer therapies promising.

### 1.2.4 Humoral adaptive responses

B cell activation results in clonal proliferation and the production of antibodies that effect anti-tumour responses in various ways including complement-mediated cell lysis, antibody-dependent cell mediated cytotoxicity (ADCC) involving NK cells and

macrophages, interference with growth or survival signals, and enhancing T cell mediated immunity through facilitated uptake and presentation of tumour antigens. The success of SEREX (serological analysis of autologous tumour antigens by recombinant cDNA expression cloning) in identifying tumour associated antibody responses has opened up the possibility of enhancing anti-tumour B cell responses *in vivo*. In B cell lymphoma, active immunisation against the unique idiotypic (Id) immunoglobulin with autologous dendritic cells (DC) vaccination and boosting with immunoglobulin conjugated to helper protein keyhole limpet hemocyanin (KLH), has resulted in T cell and humoral based anti-Id responses with clinical success (Timmerman et al., 2002). Apart from this, other approaches to *in vivo* B cell activation have not been as effective, and even potentially hazardous by inducing autoreactive antibodies. On the other hand, passive immunisation with antibodies has become an established form of tumour immunotherapy. Rituximab which binds CD20 has been shown to induce remissions in B cell lymphoma patients through ADCC (Clynes et al., 2000). Monoclonal antibodies to the Her-2/Neu receptor on breast cancer and epithelial growth factor (EGF) on epithelial tumours has provided therapeutic benefit through blocking growth signals (as reviewed by Harris (Harris, 2004). As discussed later, the renal cancer associated tumour antigen CA9 or G250 has spawned clinical trials involving monoclonal antibodies tagged to radioisotopes as a means of targeted immunotherapy (Steffens et al., 1997). As alluded to earlier, a major challenge remains in terms of improving such antibody based therapy without inducing unmanageable toxicity to normal tissues.

### 1.2.5 Cellular adaptive responses

The majority of the peripheral T cell responses to tumours are made up of the thymus-derived lymphocytes. These include the CD4 and CD8  $\alpha\beta$  TCR T cells. The optimum combination of anti-tumour cellular adaptive responses is still unresolved, but probably involves long-lasting CD8 + cytotoxic T cells (CTL) and T helper 1 (Th1) CD4 + immunity.

Naïve CD8 + T lymphocytes upon activation and co-stimulation develop into armed effectors or cytotoxic T lymphocytes (CTL) which effect tumour lysis mediated by perforin and/or Fas ligation. The pivotal role of CD8 + CTL in tumour immunity in animal models is indisputable; compelling evidence in humans stems from the frequent association between tumour progression and loss of MHC I and other components of the class I processing pathway in patients with cancer (Marincola et al., 2000). These MHC I molecules in association with tumour antigen-derived peptides form the key elements of target recognition by CTL. Accumulating evidence however, indicates that the CD4 + T cell response also plays a key role in tumour immunity. The Th1 subset of CD4 + T cells is essential for the persistence and not the induction, of CD8 + CTL responses (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). In addition, through secretion of effector cytokines such as IFN $\gamma$ , CD4 + T cells sensitise tumours to CTL lysis by upregulation of MHC I targets, stimulation of innate immunity, and inhibition of local tumour-induced angiogenesis (Mumberg et al., 1999; Qin and Blankenstein, 2000). CD4 + T cells have been shown to eradicate tumour in the absence of CD8 + T cell immunity

(Greenberg et al., 1985; Hock et al., 1991), or at least constitute the dominant effector arm in the anti-tumour response (Hung et al., 1998).

### **1.2.6 Indirect tumour targeting via the tumour vasculature**

As discussed below, tumour growth is constrained beyond 180  $\mu\text{m}$  by chronic, diffusion-limited hypoxia (Thomlinson, 1955), necessitating some form of neoplastic angiogenesis (Fidler and Ellis, 2004). Since the genetic instability and biologic heterogeneity of neoplasms are the principle causes of failure of systemic anti-tumour therapy, targeting the neovasculature of tumours by way of cytokine therapy or vaccination against tumour endothelium, has been explored as a way of attacking a genetically stable and essential component of tumours.

Type I interferons have been shown to inhibit angiogenesis through down-regulation of tumour derived factors, in a neuroblastoma model (Streck et al., 2004). There are clinical trials exploring the combined anti-angiogenic effects of IFN $\alpha$  with small molecular inhibitors of vascular endothelial growth factor (VEGF) – mediated signalling (Lara et al., 2003), and with thalidomide (Hernberg et al., 2003) in metastatic renal cell carcinoma. The essential role of IFN $\gamma$ , derived principally from activated T cells and NK cells, in effecting CD4 $^{+}$  and CD8 $^{+}$  T lymphocyte mediated anti-tumour responses through anti-angiogenesis is well described (Qin and Blankenstein, 2000; Qin et al., 2003). Tumour-associated macrophage-derived IFN $\gamma$  following T cell inactivation has been shown to be responsible for the anti-angiogenic effects of cyclophosphamide treatment, apart from its known direct cytotoxicity (Ibe et al., 2001). Several groups have

highlighted anti-tumour effects by angiogenic inhibition by other cytokines such as IL18 (Cao et al., 1999), IL12 (Duda et al., 2000; Mitola et al., 2003) and IL10 (Stearns et al., 1999).

Direct targeting of the tumour vasculature through vaccinations has opened new therapeutic possibilities. Since tumour angiogenesis is dependent on VEGF receptor -2 (VEGFR-2) expression, vaccinations by VEGFR-2 mRNA transfected (Nair et al., 2003) or peptide-loaded dendritic cells (Li et al., 2002) have been shown to result in anti-tumour effects. Recently, Tan *et al* (Tan et al., 2004) have demonstrated active tumour immunotherapy with a recombinant xenogeneic vascular target endoglin as a model antigen. High affinity antibodies have also been used to target tumour vessels (Viti et al., 1999), and have also led to enhancement of tumour immunotherapy through improvement of endogenous DC function (Gabrilovich et al., 1999).

In summary, the intricate interplay between anti-angiogenic and anti-tumour immune responses in the tumour stroma form the basis for possible synergies between the 2 seemingly divergent treatments (Cuadros et al., 2003).

### **1.3 T cell mediated anti-tumour responses and tumour antigens**

#### **1.3.1 T cell receptor (TCR) structure, activation and signalling**

The specificity and activation of T cells is conferred by their clonotypic TCR comprising two disulfide linked  $\alpha$  and  $\beta$  chains each bearing an immunoglobulin (Ig)-like constant

and variable domain, much like antibody Fab fragments. However, unlike the epitope binding domains of antibodies, the diversity of the TCR is limited by the absence of somatic hypermutation during development, and hence primarily focussed on the third complementarity determining region (CDR3) loops. The TCR engages peptide bound to surface major histocompatibility complex (MHC) on antigen presenting cells (APC), with co-receptors CD4 and 8 providing additional contact via the  $\alpha 3$  domain of the MHC. The surface expression of TCR is linked to the cytoplasmic CD3 complex, within which multiple immunoreceptor tyrosine-based activation motifs (ITAMs) exist to effect TCR signalling. Upon simultaneous engagement of peptide/MHC by TCR and co-receptor, phosphorylation of specific tyrosine residues on the CD3 ITAMs by Src family kinases Fyn and Lck leads to membrane targeting and activation of cytosolic kinase ZAP10, which induces calcium flux signals down the MAP kinase cascade, culminating in activation of transcription factors NF $\kappa$ B, NFAT and AP1. The sum of the events results in T cell proliferation, differentiation and execution of effector functions including cytokine expression and cytotoxicity.

Although specific for a particular peptide/MHC (pMHC) combination, TCR cross-reactivity is a well documented phenomenon (Mason, 1998), and has even been recognised as being essential feature for preserving efficient targeting of foreign antigens while minimising self-reactivity. However, not all variant peptides elicit the same functional T cell responses, and several studies have addressed the differential outcomes of TCR engagement depending on the quality of signal received. Peptides engaged by TCR can be broadly classified as having agonist, partial agonist or antagonist activity.



Binding of an agonist ligand to TCR triggers the full range of T cell activation including cytokine secretion, cytotoxicity and proliferation. Weak agonists act similarly, albeit requiring higher peptide concentrations and more contact time (Vidal et al., 1996). Partial agonists trigger only a subset of T cell responses (Kessler et al., 1998), whilst antagonists can suppress T cell responses to an agonist ligand (Kalergis and Nathenson, 2000). One implication from this is the use of cross-reactive altered peptide ligands, to facilitate the optimum T cell response to a tumour antigen (Clay et al., 1999; Vierboom et al., 1998). Altered peptide ligands contain specific amino acid substitutions within the native peptide which enhance the binding affinity to MHC I and hence TCR avidity, although it should be noted that it is still the lower affinity native peptide that is presented by the tumour.

### **1.3.2 Thymic selection and tolerance.**

The mature T cell repertoire is sufficiently diverse to respond with great sensitivity and specificity towards a wide array of foreign antigens. On the other hand, these same effectors are largely unresponsive to self antigens. This paradoxical requirement of T cells is fulfilled early on in their development in the thymus (Sebzda et al., 1999; Stockinger, 1999). In fact, the earlier seminal observations and proposals by Burnet (The clonal selection theory of acquired immunity in 1959) and Medawar (Billingham et al., 1953) which culminated in the 'Self/ Non-self discrimination model' (for which they shared the 1960 Nobel prize), provided the theoretical basis for the thymic selection of T lymphocytes. Here, TCR are generated by random rearrangement of TCR  $\alpha$  and  $\beta$  chain gene segments. Intrathymic education of these nascent T cells begins with survival



signals through positive selection of those T cells bearing TCR which recognise self MHC molecules on cortical epithelial cells (Bevan, 1977; Zinkernagel and Doherty, 1979), while all others undergo apoptosis or 'death by neglect' (Surh and Sprent, 1994). Negative selection meanwhile ensures the clearance of immature T cells recognising self peptide/MHC (pMHC) presented on both cortical and medullary thymic epithelium (Kappler et al., 1987; Nossal, 1994), as well as bone-marrow derived antigen presenting cells (APC) (Marrack and Kappler, 1988; Matzinger and Guerder, 1989) resulting in clonal deletion of specific subsets of self reactive T cells. That the same TCR can mediate both positive and negative selection, is reconciled through the affinity model of thymic selection. Several *in vitro* and *in vivo* studies have confirmed this model by demonstrating that low affinity TCR-p/MHC interactions result in survival or positive selection, whilst high affinity interactions lead to negative selection (Alam et al., 1996; Hogquist et al., 1994; Lee et al., 1999a; Liu et al., 1995; Williams et al., 1999). The mature T cells emerging from the thymus that are potentially reactive to peripherally expressed self antigens are therefore of 2 forms; higher avidity T cells recognising self antigens that were absent from the thymus (or expressed in low amounts), or those that survive negative selection, and are therefore necessarily of low avidity. This pattern of T cell development has key implications for T cell based tumour immunotherapy, as exemplified by the data presented in Chapter 5.

Obviously, the general rarity of autoimmune phenomenon in healthy individuals implicates some form of peripheral tolerance mechanism to avoid self-reactivity. These include peripheral clonal elimination (Jones et al., 1990; Rocha and von Boehmer, 1991;

Webb et al., 1990), induction of T cell unresponsiveness or anergy (Morahan et al., 1989; Ramsdell et al., 1989), downregulation of TCR and/or co-receptors (Schonrich et al., 1991), immunological ignorance (Ohashi et al., 1991) and more recently described, suppression by T regulatory cells (Sakaguchi, 2000; Shevach, 2000). The deletion or induction of anergy in self-reactive T cells in the periphery has been suggested to involve uptake of self-antigens by bone marrow-derived APC (as in the thymus) that co-localise with these naïve T cells in the draining lymph nodes (Kurts et al., 1997). Standing on the shoulders of the 'Self/Non-self model', the recently proposed 'Danger hypothesis' has added yet another layer of complexity to the issue of immune discrimination and activation (Matzinger, 1998; Matzinger, 2002). Apart from tolerance mechanisms both central and peripheral, the lack of self-reactivity and tumour immunogenicity in most cases is attributed to the absence of 'danger' signals (proinflammatory cytokines including tumour necrosis factor, IFN $\gamma$ ; foreign pathogens, necrotic tissue) in the micro-environment, leading to suboptimal T cell activation by dendritic cells, anergy and deletion.

### **1.3.3 Tumour antigens**

The initial step towards establishing the relevance of antigen specific T cell responses in cancer immunology was taken by Van Der Bruggen and colleagues who identified a gene encoding an antigen recognised by CTL on a human melanoma (van der Bruggen et al., 1991). Since then, the field of T cell based tumour immunotherapy has been largely driven by the hunt for relevant and clinically useful tumour antigens.

Tumour antigens can broadly be classified into patient or tumour specific antigens, and shared antigens (Gilboa, 1999). Tumour specific antigens derive from unique mutations associated with the malignant phenotype (Lengauer et al., 1998). While being exclusive to the tumour and absent from normal tissue, these antigens require patient (and tumour) specific vaccines to target these unique antigens. Additionally, as the cancer progresses, selection pressures continually modify the tumour phenotype, rendering previous antigens irrelevant for current therapy. Tumour specific antigens can also arise from oncogenic viral antigens, as exemplified by the E7 oncoprotein of the human papillomavirus, present in 95% of cervical cancer lesions (zur Hausen, 2000). Being essentially foreign antigens without all the constraints of central and peripheral tolerance mechanisms, effective anti-tumour T cell responses have been realised in several clinical trials (Adams et al., 2001; Muderspach et al., 2000). The second category of shared tumour antigens comprise non-mutated antigens expressed both in tumour and normal tissue. Based on tissue expression profiles, this heterogenous group include tissue specific/ differentiation antigens and ubiquitously expressed antigens. The former group include melanoma specific antigens eg. MART-1, gp 100 and tyrosinase (Brichard et al., 1993; Kawakami and Rosenberg, 1997), and the cancer-testis group of antigens such as MAGE-1 and NY-ESO (Wang et al., 1998). Ubiquitously expressed antigens include p53 and telomerase, and the oncofetal antigens like carcinoembryonic antigen (CEA) (Berinstein, 2002). These antigens are overexpressed in tumours with comparably low or negligent expression in normal tissue.

The majority of tumour antigens are overexpressed ubiquitous self-antigens that find expression both in the thymus and periphery. The T cell repertoire available for tumour immunotherapy against such antigens is significantly affected by central tolerance mechanisms, and characteristically of low avidity (de Visser et al., 2000). Notwithstanding, ubiquitously expressed tumour antigens are attractive candidates for tumour immunotherapy because of their shared expression amongst many tumour types, thus facilitating the development of vaccines with broad applicability. Examples of these include p53 protein which is upregulated in over 50% of tumours (Levine et al., 1991; Theobald et al., 1995), telomerase (Vonderheide et al., 1999) which finds strong expression in 85% of human cancers, and of relevance to this thesis, carbonic anhydrase isoenzyme 9 (CA9 or G250) which is discussed further in Chapter 4 and 5. The inherent low avidity of such self-reactive T cells has been shown not to be an insurmountable hurdle in terms of effecting anti-tumour immunity (Cordaro et al., 2002), and even considered desirable by way of avoiding autoimmune side effects (Morgan et al., 1998; Speiser et al., 1997).

## **1.4 Dendritic cells and tumour antigen presentation**

### **1.4.1 Antigen presentation**

In the direct priming of CD8<sup>+</sup> T cells, endogenous nascent proteins transcribed and translated from within the APC are first processed via the ubiquitin-proteasomal pathway, with ensuing 8-10 residue peptides transported from the cytosol into the endoplasmic reticulum (ER) through the mediation of transporter associated with antigen presentation

(TAP), loaded onto MHC class I aided by the chaperon tapasin, channelled through the Golgi apparatus and on to the cell surface. Exogenous antigens are taken up into endosomes, degraded in situ into polypeptides and directly loaded onto nascent MHC class II within MHC II-rich compartments for cell surface expression without entering the cytosolic space. These peptide/MHC II are thus presented to CD4+ T cells. A fraction of peptides are loaded onto empty MHC II molecules recycled from the cell surface. Exogenous antigens also find their way into the MHC I pathway through the process of 'cross presentation', a pathway of emerging importance to the understanding of tumours-induced T cell immunity.

Three distinct proteolytic processes have been shown to be important for MHC class I antigen processing and presentation (Rock et al., 2002). Ubiquitinated proteins are degraded by the 26S proteasome into oligopeptides, followed by trimming by cytosolic aminopeptidases into antigenic peptides of correct lengths for MHC I loading. Counterbalancing this pathway of antigenic peptide generation, proteasomal products are also overwhelmingly subject to a third fate of destruction by endo and exo-peptidases. Interferon  $\gamma$  (IFN $\gamma$ ) alters this pathway through induction of 3 novel proteins; MHC encoded proteins LMP2 and LMP7 (Glynne et al., 1991; Martinez and Monaco, 1993; Ortiz-Navarrete et al., 1991), and MECL1 lying outside of the MHC locus (Hisamatsu et al., 1996), which modify proteasomal activity. This altered proteasome, sometimes referred to as the 'immunoproteasome' potentially generates a distinct set of antigens from the standard proteasome. In vitro studies performed with viral epitopes has suggested that the immunoproteasome is more effective in processing some antigenic

peptides (Cerundolo et al., 1995). In contrast, work by Morel *et al* (Morel et al., 2000) demonstrated that by being less efficient than the standard proteasome, dendritic cells (DC) which constitutively express the immunoproteasome can therefore fail to present certain peptide antigens, leading to relevant CTL escape from thymic deletion and failure of peripheral activation. Besides being proposed as a mechanism of minimising autoimmunity in the face of peripheral self-reactive T cells that had eluded thymic deletion, the implications of the immunoproteasome extend to tumour evasion and immunotherapy. Antigen processing by DC-associated immunoproteasome could prime T cells that are irrelevant in terms of actual tumour antigen recognition. Systemic IFN $\gamma$  therapy could similarly modify antigen processing and account for some observations of tumour progression after such treatment.

#### **1.4.2 Dendritic cells**

Dendritic cells (DC) are professional APC that provide potent stimulation of naïve B and T cells, and form the bridge between the acquired and innate immune response, the latter through its germ line encoded pattern recognition receptors (PRR) that engage conserved pathogen-associated molecular patterns (PAMP). By presenting endogenously generated as well as exogenously acquired foreign antigen on cell surface MHC class I and II molecules respectively (signal one), together with co-stimulatory molecules such as CD80 and 86 (signal two), DC efficiently prime naïve T cells, a paradigm first described by Bretscher and Cohn in their original ‘2 signal hypothesis model’ (Bretscher and Cohn, 1968). It has emerged that additional signals by way of CD4 T helper cell mediated CD40 ligation of DC are also important for subsequent CD8 T cell activation (Bennett et al.,

1998; Ridge et al., 1998; Schoenberger et al., 1998), although not absolutely necessary (Young and Steinman, 1990). Recently, other accessory signals by way of OX40 and IL12 have been described as being important to the DC activation of CD4 and CD8 T cell responses (De Smedt et al., 2002; Valenzuela et al., 2002).

Newly generated DC migrate from their origin in the bone marrow via the blood circulation to reside in peripheral extra lymphatic tissue as immature DC, where they play out their roles as immune sentinels (Banchereau et al., 2000). These myeloid CD34<sup>+</sup> DC progenitors exist in the blood in two forms; CD14<sup>+</sup>/CD11c<sup>+</sup> and CD14<sup>-</sup>/CD11c<sup>+</sup> monocytes, the former differentiating into interstitial DC under the in vitro influence of GM-CSF and IL4, and the latter into Langerhans DC with GM-CSF, IL4 and TGFβ. Immature DC are efficient in antigen capture, where upon they migrate into draining lymphoid organs, all the while undergoing phenotypic and functional changes that culminate in the complete transition into an APC. Numerous factors induce the maturation of DC including pathogen related molecules such as bacterial lipopolysaccharide (LPS), cytosine-phosphate guanosine (CpG) sequence motifs, viral double stranded RNA, proinflammatory cytokines, and T cell derived signals such as CD40L. The mature DC is primed for its new function as an APC to naïve T cells in the lymphoid tissue through the loss of endocytic/ phagocytic receptors, upregulation of co-stimulatory molecules CD80 and 86, morphological changes into its eponymous form, and upregulation of MHC I and II expression.



### 1.4.3 Cross presentation of tumour antigens

As alluded to earlier, the induction of peripheral tolerance and deletion of self-reactive T cells involves the phenomenon of cross presentation (or cross tolerance in this case) by bone marrow derived APC. The term 'cross priming' was first coined by Bevan (Bevan, 1976a; Bevan, 1976b) in reference to the ability of host APC to prime CTL responses against minor histocompatibility antigens captured from foreign donor cells. The term 'cross presentation/ tolerance' has subsequently been used to describe the uptake and re-presentation of exogenous cell-associated antigens primarily in the MHC class I pathway. Since naïve self-reactive T cell migration from the thymus is restricted to the secondary lymphoid organs, peripheral tolerance requires that bone marrow derived APC carrying exogenous self-antigens ingested from peripheral tissues contacts with such naïve T cells in lymphoid tissue, processes and cross presents the cognate peptide complexed to MHC I to the corresponding TCR (Kurts et al., 1997). In much the same way, peripherally expressed extra-lymphatic tumour antigens are taken up by the same bone marrow derived APC which co-localise with, and cross present to naïve T cells (Huang et al., 1994). The key cross presenting APC *in vivo* has been identified as the dendritic cell, the specific subtype of which is still debated although the CD8 $\alpha$ <sup>+</sup> DC subtype has been shown to be especially important (den Haan et al., 2000; Pooley et al., 2001; Smith et al., 2003). Other types of endocytic cells like macrophages and B cells have also been shown to have a similar albeit less efficient cross presenting capability *in vitro* (Reis e Sousa and Germain, 1995). Three recent papers corroborate the idea that cellular proteins (as opposed to peptides bound to heat shock protein chaperons) are the main molecular currency of cross presentation (Norbury et al., 2004; Shen and Rock, 2004; Wolkers et



al., 2004). The uptake and processing by DC of such antigens by phagocytosis is expedited by the direct association of the ER membrane with nascent phagosomes, allowing a crucial intersection of the endosomal system with the ER, the classical site of MHC I peptide loading (Ackerman et al., 2003; Houde et al., 2003). Being potentially capable of both cross priming and cross tolerance, it is the maturation and activation state of the DC that ultimately determines the fate of the naïve T cell encountered (Moser, 2003).

The debate on the actual relevance of *in vivo* cross presentation of tumour antigens continues. Zinkernagel and co-workers have argued that in the absence of powerful DC activating signals such as in infections, cross presentation of tumour antigens must necessarily be inefficient and therefore irrelevant to anti-tumour immunity (Ochsenbein et al., 2001; Zinkernagel, 2002). Instead, extra-lymphatic tumours are largely ignored by the immune system, unless direct presentation by tumours infiltrating secondary lymphoid organs occurs. Melief (Melief, 2003) has posited a counter argument that cross presentation is the norm and direct presentation the exception, by noting that TCR-transgenic T cells in mice did not recognise tumour antigen in draining lymph nodes in the absence of TAP. Significantly, addition of DC activating signals like CD40 agonist antibody led to priming of effective anti-tumour T cell responses (van Mierlo et al., 2002). Using a tumour model of spontaneously arising insulinomas expressing a defined tumour antigen, the group led by Ohashi (Nguyen et al., 2002) reported that advancing tumour growth enhanced cross presentation of tumour antigens, with potent T cell mediated anti-tumour immunity elicited by administration of tumour peptide with CD40

agonist antibody, or by viral immunisation. Nowak *et al* (Nowak et al., 2003) has suggested that cellular proteins released during tumour cell apoptosis *in vivo* enhances tumour antigen cross priming of host tumour specific CD8+ T cells. The cross presentation pathway has also been suggested to be relevant to induction of superior anti-tumour T cell responses by vaccination, since Li *et al* have shown that cell-associated antigens activate CD8+ T cells *in vivo* more efficiently than soluble antigen (Li et al., 2001b). Anti-tumour gene therapy by way of DNA vaccines relies primarily on the cross priming effect whereby antigen released by muscle cells is taken up and cross presented by bone marrow derived DC (Corr et al., 1996; Iwasaki et al., 1997). On balance therefore, the evidence seems to weigh towards a key role of the bone marrow derived DC and cross presentation of tumour antigens in effecting an anti-tumour T cell response. Indeed, the evolutionary importance of cross presentation in adaptive immunity in general has been implicated by the recent finding of a tyrosine-based targeting signal in the cytoplasmic tail of MHC I which was critical for routing MHC I molecules into the endolysosomal compartment of DC (Lizee et al., 2003).

### **1.5 Tumour escape mechanisms to T cell immunity**

The understanding of tumour-host interactions remains elusive despite significant progress in the identification of tumour antigens recognised by autologous T cells. In particular, most human tumours do not regress and continue to grow in spite of spontaneous or vaccination-induced immune responses demonstrated in circulating lymphocytes.

MHC I restricted cytotoxic T lymphocytes (CTL) are considered a major effector of anti-tumour immunity. Therefore loss or down regulation of MHC I expression is a common theme in tumour escape (Garcia-Lora et al., 2003), and correlated to poorer clinical outcomes (Kageshita et al., 1999). On the other hand, down regulation of MHC I has sometimes been associated with a better outcome, possibly related to the effects of NK mediated cytotoxicity in the absence of HLA expression (Menon et al., 2002). Besides MHC I itself, the processing of endogenous antigens in tumour cells can also be affected, especially those components related to peptide transport (Seliger et al., 2003). Evasion of immune recognition can also result from immunodominance which biases the host response to an epitope while antigen loss variants in the tumour accumulate (Schreiber et al., 2002). Perhaps the most powerful circumstantial evidence for this derives from extensive clinical studies showing loss of antigen expression in lesions from patients vaccinated against that same antigen (Riker et al., 1999).

Tumours can actively suppress the immune response. It has been known for years that sera from cancer patients contain a plethora of immune suppressive proteins, including adhesion molecules (Sanchez-Rovira et al., 1998), gangliosides (Peguet-Navarro et al., 2003), soluble Fas (Tsutsumi et al., 2000) and cytokines, principally TGF- $\beta$  (Wahl et al., 2004) and IL10 (Mocellin et al., 2003). Up regulation of MHC related molecules as HLA-E and HLA-G can also lead to suppressive effects via ligation of inhibitory receptors (Cerwenka and Lanier, 2003; Chouaib et al., 2002) and poorer prognoses (Ugurel et al., 2001). Recently, the CD4<sup>+</sup>/CD25<sup>+</sup> T regulatory cell has been proposed as an important means of immune suppression (Sakaguchi et al., 2001). Consistent with

this, Shimizu has reported that tumour unresponsiveness in mice can be reversed through depletion of CD25 + / CD4 + T cells in mice prior to tumour challenge (Shimizu et al., 1999).

Apart from suppression, tumour escape can take a more drastic form by actual deletion of tumour reactive T cells, or at least render them anergic. Central tolerance to high avidity T cells results in their deletion from the immune repertoire, since many tumour antigens are self-antigens that are expressed in the thymus (Lauritzsen et al., 1998). Peripheral deletion of tumour reactive T cells has been attributed to the expression of Fas ligand on tumour cells concomitant with upregulation of Fas on activated T cells (Reichmann, 2002), but this has been challenged (Restifo, 2000). Induction of antigen-specific T cell anergy has been shown to be an early event in the course of tumour progression (Staveley-O'Carroll et al., 1998). Even more intriguing, vaccination with immunogenic peptides derived from tumour antigens could have an enhancing effect on tumour growth by inducing T cell anergy (Toes et al., 1996).

T cell defects in signal transduction represent yet another means of tumour escape (Mizoguchi et al., 1992; Radoja and Frey, 2000), and this has been confirmed in a wide variety of cancers (Whiteside, 1999). Interestingly, surgical resection of the primary tumour has been shown to reverse tumour-induced immune suppression of both cellular and humoral in a mouse mammary tumour model (Danna et al., 2004). Tumour associated macrophages (TAM) have been extensively described to suppress immune responses in tumour bearing hosts (Alleva et al., 1995; Mantovani et al., 1992). The

effect has been ascribed to the production of reactive oxygen metabolites by TAM which cause stress-induced CD3  $\zeta$  loss and induction of apoptosis in lymphocytes (Gastman et al., 1999; Kono et al., 1996). It has been suggested that the immune system itself has a bipolar effect on tumours, both inhibiting and promoting growth (Guindi, 2000; Prehn, 1994). Stewart *et al* (Stewart et al., 1995) observed a reduced risk of breast cancer in a large cohort of women with heart or kidney transplants on immunosuppression. Cytokine production (eg IL4) during the immune response could be responsible for promoting tumour growth through inhibition of apoptosis (Dancescu et al., 1992), as could pro-angiogenic factors from tumour infiltrating lymphocytes (TIL) like vascular endothelial growth factor (VEGF) (Freeman et al., 1995). Indeed, even more subversive action by tumours have included the expression of IL2 receptors for their growth (Capelli et al., 1999) and their association with increased drug resistance (Kuhn et al., 2003).

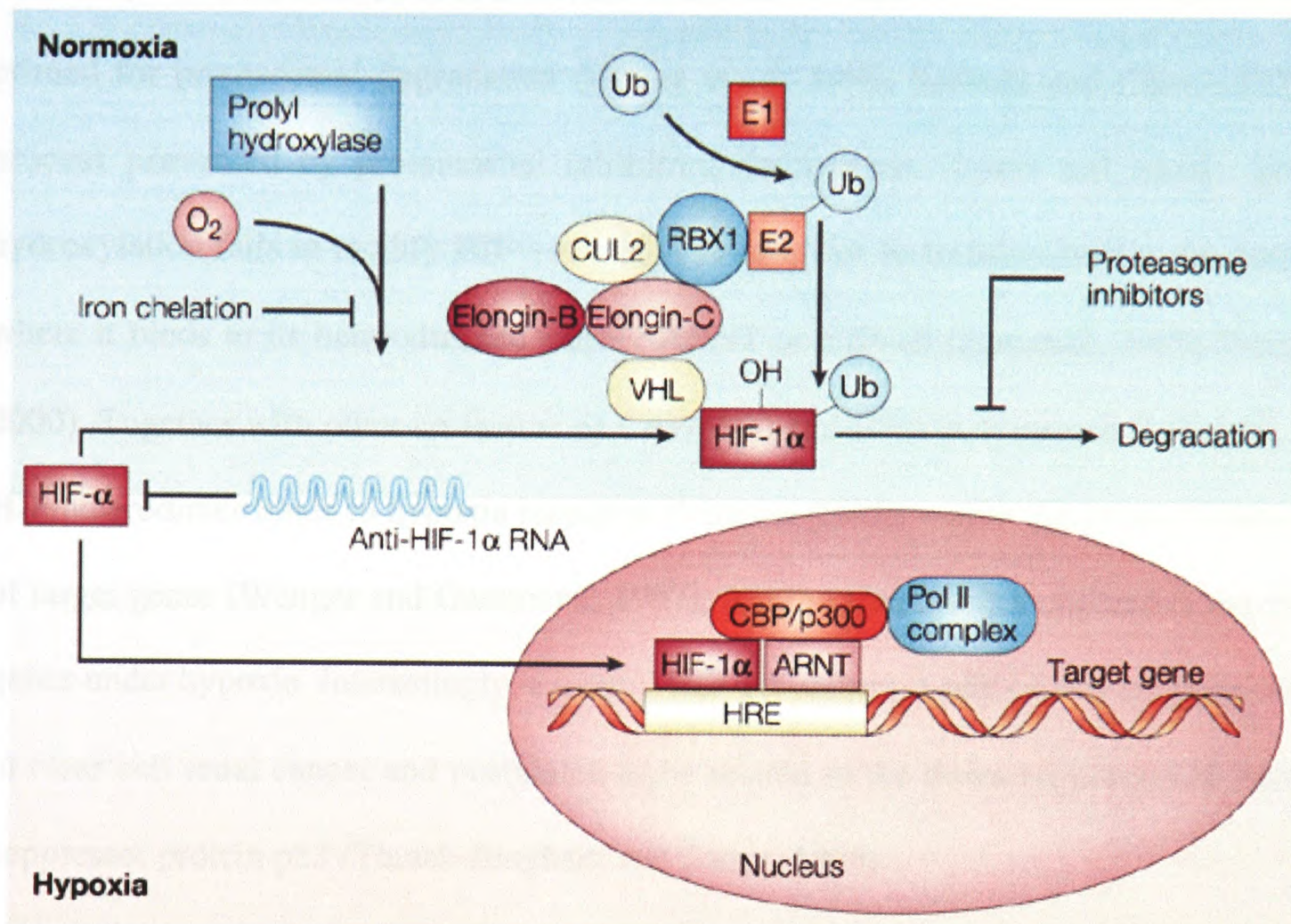
In summary, tumour escape represents the final hurdle to formulating effective cancer immunotherapy, but given the immense plasticity of the tumour cell, and the complex interplay between tumour, effector T cell and the microenvironment, this continues to pose a major challenge.

### **1.6 Tumour hypoxia**

Mammalian cells have evolved to adapt to hypoxic environments by increased expression of specific hypoxia- inducible genes. The expression of these genes include such adaptive responses as increased glucose uptake and glycolysis, vasodilation, erythropoiesis and angiogenesis, balanced against the induction of stress-response and apoptotic pathways



(Semenza, 2001). A key player in this repertoire of responses is hypoxia-inducible factor (HIF), a heterodimeric transcription factor containing a cytoplasmic oxygen labile  $\alpha$  subunit (HIF-1 $\alpha$ ), and a constitutively expressed nuclear  $\beta$  subunit, variously known as aryl hydrocarbon nuclear translocator (ARNT) or HIF-1 $\beta$  (Wang et al., 1995).



**Figure taken from : Hypoxia – a key regulatory factor in tumour growth.**  
**Harris AL. Nature Reviews/ Cancer Jan 2002. Vol 2; 38.**

As depicted in the above figure, HIF-1 $\alpha$  is the key orchestrator of the hypoxia response. Under conditions of normoxia (upper blue panel), it is post-transcriptionally modified by the enzyme prolyl hydroxylase (Jaakkola et al., 2001) which requires oxygen, ferrous iron and 2-oxoglutarate, to convert HIF-1 $\alpha$  into its hydroxylated form. To date there are 3 known prolyl hydroxylases (Bruick and McKnight, 2001; Epstein et al., 2001), the

significance of which remains to be elucidated. Iron chelation inhibits prolyl hydroxylase activity via its iron moiety, leading to stabilisation of HIF-1 $\alpha$ . Otherwise, hydroxylated HIF-1 $\alpha$  interacts with the von Hippel Lindau (VHL) ubiquitin-ligase complex consisting of VHL, elongin-B and C, CUL2, RBX1 and an ubiquitin-conjugating enzyme E2. The complex, together with ubiquitin-activating enzyme E1 mediates the ubiquitination of HIF-1 $\alpha$  (Cockman et al., 2000; Maxwell et al., 1999; Ohh et al., 2000), which is thus primed for proteasomal degradation (Huang et al., 1996; Salceda and Caro, 1997), a process prevented by proteasomal inhibitors. In hypoxia (lower red panel), prolyl hydroxylation fails to modify HIF-1 $\alpha$ , which is then free to translocate into the nucleus where it binds to its heterodimeric partner ARNT or HIF-1 $\beta$  (Semenza, 1998; Wenger, 2000). Together with other co-factors as CBP/p300 and DNA polymerase II (Pol II), the HIF heterodimer binds to hypoxia response elements (HRE) within the promoter regions of target genes (Wenger and Gassmann, 1997), thereby transcriptionally activating these genes under hypoxia. Interestingly, a natural HIF-1 $\alpha$  antisense mRNA has been described in clear cell renal cancer and postulated to be related to the down regulation of tumour suppressor protein p53 (Thrash-Bingham and Tartof, 1999).

HIF - 2 $\alpha$  is a recently identified hypoxia inducible transcription factor, with 48% sequence homology to HIF -1 $\alpha$  (Wenger, 2000). Like HIF -1 $\alpha$ , its protein levels are low in normoxia, but increase during hypoxia and form functional heterodimers with HIF -1 $\beta$  which bind to HRE of target genes. Despite high sequence homology and functional similarities, their downstream effects are very different. HIF-1 $\alpha$  has a critical role in embryogenesis. Mouse embryos homozygous for HIF -1 $\alpha$  deletion have abnormal

cardiac and vascular development leading to embryonic lethality (Minet et al., 2000). HIF-2 $\alpha$  knock out mice have normal circulatory system development, but perish from bradycardia at mid-gestation, possibly because HIF-2 $\alpha$  is implicated in the transcriptional regulation of catecholamine synthesis (Knies, 2000).

Unlike normal physiological adaptations to hypoxia, tumours have evolved a strategy which subverts this mechanism to further their own survival and proliferation. The seminal observations by Vaupel *et al* (Hockel et al., 1999; Hockel and Vaupel, 2001; Vaupel et al., 2001) on intra-tumoral oxygenation using oxygen sensing electrodes, revealed that low oxygen tensions in cancers were common and associated with metastatic potential with overall poorer survival. In one study on breast tumours, the median oxygen tension in normal tissue was 8%, compared with 4% or even less than 1% in tumour bearing areas. Lartigau reported that metastatic melanoma lesions had a mean oxygen tension of 1% compared with 5% in adjacent normal tissue (Lartigau et al., 1997). Brizel (Brizel et al., 1996) reported an increased likelihood of distant metastasis in human soft tissue sarcomas with tumour hypoxia. Interestingly, Tomes (Tomes et al., 2003) demonstrated that within the tumour mass, stroma associated hypoxia had a better prognosis than epithelial hypoxia in invasive ductal carcinomas of the breast, perhaps related to the preferential expression of HIF-2 $\alpha$  in tumour stromal macrophages (Talks et al., 2000). The results implicate different responses to hypoxia between tumour epithelium and stroma, this being of pertinence to HIF-directed anti-tumour therapies. There have been described two etiologic patterns of tumour hypoxia *in vivo* (Vaupel et al., 2001). Chronic hypoxia has been attributed to the diffusion limits of oxygen within



tissue, reported as 180  $\mu\text{m}$  from adjacent blood vessels (Thomlinson, 1955). Uncontrolled tumour growth causes tumours to rapidly extend beyond these limits. Acute hypoxia occurs when aberrant blood vessels shut down and shunt blood away from the tumour tissue, causing a perfusion-limited type of hypoxia. Resistance to radiotherapy has been suggested to be due to persistent tumour proliferation and survival in the former in contrast to the latter (Wouters and Brown, 1997).

The reason for the existence and persistence of hypoxia within the tumour mass has been attributed to an imbalance between supply and demand; metabolically active tumour cells deplete extracellular oxygen, while aberrant tumour vasculature with erratic blood flows reduce oxygen supply (Brown and Giaccia, 1998). Tumour hypoxia leads to hypoxia inducible gene effects through the action of HIF-1 as described, which translate into a plethora of biological processes relevant to cancer growth and metastasis, including cell proliferation, migration, altered metabolism, angiogenesis, apoptosis and immortalisation (Harris, 2002). Apart from HIF, tumour hypoxia activates other transcription factors including cyclic AMP response element binding gene (CREB) (Taylor et al., 2000), nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) (Schmedtje et al., 1997), early growth response (EGR-1) (Yan et al., 2000) and placental growth factor (PIGF) (Carmeliet et al., 2001). Tumour associated thrombosis has been ascribed to EGR-1, while PIGF has been shown to amplify vascular endothelial growth factor (VEGF) signalling in cancer cells. Conversely, apart from hypoxia, several oncogenic pathways intersect at HIF (Harris, 2002). Thus, within the hypoxic milieu, tumour cells continually evolve through their inherent genetic instability, taking advantage of growth promoting and anti-apoptotic

signals while selecting against those susceptible to growth inhibition and apoptosis, until a more aggressive tumour phenotype emerges.

In conclusion, tissue hypoxia is a unique and widespread phenomenon in tumours, with limited and transient occurrences in normal tissue. Tumour hypoxia induces hypoxia-regulated gene expression, many of which play key roles in the malignant transformation and selection for aggressive tumour phenotypes. Therefore, it is reasonable to speculate whether such an inherently important tumour specific phenomenon could lend itself to being targeted by a tumour specific therapy such as T cell based immunotherapy. Since these hypoxia-regulated proteins are self-antigens that are ubiquitously expressed in most tissues including the thymus, T cell immunotherapy against such antigens will have to surmount the problem of intrinsically low avidity T cells available for therapy. This could potentially be overcome with more efficiently triggering of low avidity T cells with higher affinity variant peptides, bearing in mind that the antigens expressed on the tumour inevitably remain low affinity. Alternatively, induced over-expression of these target antigens on tumour cells could compensate for low avidity T cell effectors, such as by hypoxic induction of hypoxia-regulated antigens. Apart from inherent tumour hypoxia, augmentation of the hypoxia response through chemical means such as iron chelation, or in synergy with anti-angiogenic therapies which target the tumour vasculature, could be effectively combined with hypoxia-directed T cell immunotherapy. This thesis aims to explore the possibilities of targeting the hypoxic tumour phenotype with specific T cell based immunotherapy, as set forth in the following objectives:

### Chapter 3. A hypoxia regulated peptide as a tumour antigen in a mouse model

- to establish a working model of a hypoxia regulated antigen in vitro and in vivo
- to analyse the immunogenicity of a hypoxia regulated antigen
- to determine the functionality of this hypoxia regulated antigen as a tumour rejection antigen

### Chapter 4. Human CA9/G250 specific T cell responses in a transgenic mouse model

- to determine the utility and specificity of novel HLA-A2K<sup>b</sup> and A2/CA9 tetramers
- to characterise the interactions of transgenic murine CD8<sup>+</sup> T lymphocytes with endogenously and exogenously presented human CA9 peptide

### Chapter 5. Human CA9/G250 specific T cell responses in healthy donors and Renal cancer patients

- to compare the natural CA9 specific T cell responses in patients and healthy individuals using novel HLA-A2/CA9 tetramers
- to correlate these CA9 responses with clinical data
- to characterise the functionality and avidity of these CA9 reactive CD8<sup>+</sup> T lymphocytes

### Chapter 6. Synergistic anti-tumour effect of metronomic cyclophosphamide and specific immunotherapy in a mouse model

- to determine if metronomic dosing of cytotoxic drug cyclophosphamide could be effectively combined with specific immunotherapy to effect tumour rejection

- to elucidate the kinetics of immune suppression with metronomic dosing

Chapter 7. Inducing expression of hypoxia regulated proteins in tumour cell lines with a novel iron chelator desferri-exochelin

- to compare desferri-exochelin with desferrioxamine in the induction of hypoxia regulated proteins through HIF
- to determine if desferri-exochelin could effect an anti-angiogenic response in vitro

## Chapter 2. Materials and Methods

### MATERIALS

#### 2.1 Cell lines and media

- **B16.F10** murine melanoma cell line were obtained from the Cancer Research UK cell services. **B16.F10*mel3*** melanoma cells (C57BL/6, H-2<sup>b</sup>) were modified to express the *mel3* polyepitope construct in combination with enhanced green fluorescent protein from a bicistronic mRNA (pIRES2-EGFP vector, Clontech, Basingstoke, UK) (Prevost-Blondel et al., 1998). The *mel3* construct consists of a string of five HLA-A2 and two HLA-A1 melanoma epitopes, and the influenza nucleoprotein epitope restricted by H-2D<sup>b</sup> (ASNENMDAM) (Palmowski et al., 2002). **B16.F10*gp33*** melanoma model expressing a minigene encoding the H-2D<sup>b</sup>-binding peptide from LCMV glycoprotein (GP<sub>33-41</sub>) was also used. B16.F10*mel3* and B16.F10*gp33* were provided by Dr IF Hermans, and cells were passaged in media containing G-418 antibiotic (Geneticin, Gibco, Paisley, UK) at 1mg/ml.

- **Z16** The  $\beta$ gal gene under the control of a hypoxia-responsive promoter derived from the murine phosphoglyceratekinase (PGK) gene and SV40 promoter, was stably transfected into a B16.F10 murine melanoma cell line. All cells were passaged in media containing G-418 antibiotic at 1.8 mg/ml. Z16 were a kind gift from Prof Ian Stratford, Dept of Pharmacology, University of Manchester.

- **C4.5 and Ka13** Chinese hamster ovary (C4.5) and a mutant clone Ka13 which does not express HIF were developed by the group of Dr Peter Ratcliffe, Wellcome Trust, Oxford (Wood et al., 1998).
- **HuDMEC** Human dermal microvascular endothelial cells were obtained from the Cancer Research UK cell services, and passaged at confluence in MCDB131 media (cell services) supplemented with 10% FCS, glutamine, heparin (5 IU) and 30 ug/ml endothelial cell growth supplement (Sigma, Dorset, UK)
- **RCC4** Human HLA-A2 renal cell carcinoma line (clear cell variant) was obtained from the Cancer Research UK cell services.
- **JY** Human lymphoblastoid B cell line (LCL) homozygous for HLA-A2 were provided by Dr M Salio.
- **.221A2** Wild type .221 cells are a mutated LCL with complete absence of HLA-A,B and C expression (Shimizu and DeMars, 1989). Transfected MHC I genes are expressed in amounts similar to the endogenous genes, cell surface expression being limited by post-translational processes. .221A2 LCL were stably transfected and expressed HLA-A2 with absence of any other MHC I. The cells were provided by Dr J Mongkulsapaya.
- **MDA468** (ATCC HTB-131), a line from human metastatic adenocarcinoma of the breast, were obtained from the Cancer Research UK cell services, and passaged at

subconfluence once a week in E4 media supplemented with 10% fetal calf serum and glutamine.

- **Media.** Unless stated otherwise, all cell cultures were passaged in R10 comprising RPMI (Sigma-Aldrich , Dorset, England) with 2 mM glutamine, 1 % penicillin-streptomycin,  $5 \times 10^{-5}$  M 2-mercapto-ethanol (all Invitrogen Ltd, Paisley, UK) and 10 % fetal bovine serum (Globepharm, Guildford, England).

## **2.2 DNA oligonucleotides and peptides**

- **Oligonucleotides** were obtained commercially through Invitrogen Ltd, Paisley, UK. For the generation of recombinant plasmid containing the CA9<sub>254-262</sub> minigene construct, sense and anti-sense oligonucleotide strands comprising the relevant gene segment with a Kozak consensus sequence upstream and flanked by Bgl II and Not I restriction sites were annealed together:

5' GATCTCACCATGCACCTCAGCACCGCCTTTGCCAGAGTTTAGC 3'

3' AGTGGTACGTGGAGTCGTGGCGGAAACGGTCTCAAATCGCCGG 5'

- **DNA primers** for sequencing of CA9 gene insert were manufactured by Cancer Research UK research services.

- **Peptides** used for generating tetramers and for vaccination procedures were generated by Cancer Research UK peptide services, and also by the Human Immunology Unit,



Oxford. Peptides were dissolved in dimethyl sulphoxide (DMSO) (Sigma, Dorset, UK); to 10 or 50 mM concentrations and stored at  $-20^{\circ}\text{C}$ .

### **2.3 Tetramers**

Unless stated otherwise, all tetramers were generated in-house by the author, using the method described by Altman (Altman et al., 1996). Recombinant proteins of MHC I heavy chain containing the BirA-substrate peptide (BSP, LGGIFEAMKMELRD) at the C-terminus, and human  $\beta 2$  microglobulin ( $\beta 2\text{m}$ ) were expressed as inclusion bodies and purified from transformed E coli bacteria (DH5 $\alpha$ ). The bacterial stock was provided by Ms D Sheppard. Initially, transformed bacteria were inoculated in low salt LB media to a density of 0.4 OD<sup>600</sup>, and induced with 0.5ml/L of 1M IPTG. The culture was pelleted after 4 hours induction and resuspended in ice-cold PBS. Efficiency of induction was assessed by comparing bands following separation of protein solution on SDS-PAGE gels stained with Coomassie blue. Purification of inclusion bodies was performed by sonication of bacterial pellets and centrifugation. After discarding the clear supernatants, pellets of bacterial debris were homogenised manually, washed with Triton buffer and resuspended in 8M cold urea solution. Refolding of the peptide-MHC (pMHC) monomer was achieved by combination of peptide,  $\beta 2\text{m}$  and heavy chain in cold refolding buffer with continuous stirring at  $4^{\circ}\text{C}$  for 48 hours. The solution was concentrated down to about 7 mls by using ultrafiltration columns (Amicon, UK) or nitrogen gas pressurised stir-cells through a 10kDa ultrafiltration membrane (Millipore, UK). Superdex-75 FPLC columns (Pharmacia, UK) were used to isolate refolded pMHC monomers at elution volume of 160 mls. Enzymatic biotinylation of the BirA site of the monomer was



achieved by overnight incubation with BirA enzyme at room temperature. Biotinylated complexes were isolated through a similar FPLC setup and concentrated down with Centriprep 10 columns (Amicon, UK). Monomers were stored at  $-20^{\circ}\text{C}$  before tetramerization with fluorescence-labelled streptavidin (Extravidin-R-Phycoerythrin conjugate; Sigma, Dorset, UK). Successful tetramerization was monitored by biotin ELISA assay.

- **H-2K<sup>b</sup>/βgalactosidase tetramer** for βgal H-2 K<sup>b</sup> restricted peptide βgal<sub>96-103</sub> DAPIYTNV
- **HLA-A2/CA9 tetramers** for four CA9 derived peptides (CA9<sub>26-35</sub> LLSLLLLMPV, CA9<sub>254-262</sub> HLSTAFARV, CA9<sub>271-279</sub> GLAVLAAFL, CA9<sub>421-429</sub> GLLFAVTSV)
- **HLA-A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer** for CA9 derived peptide (CA9<sub>254-262</sub> HLSTAFARV)
- **H-2 D<sup>b</sup>/ NP<sub>366-374</sub> tetramer** was provided by Dr MJ Palmowski

#### **2.4 Recombinant plasmids and viruses**

- **pSG2/CA9 and pSC11/CA9** Recombinant plasmids encoding for human CA9 were obtained by cloning the 1.5kb segment of CA9 from pSC5C (a gift from Dr Pastorek, Slovak Academy of Sciences, Slovak Republic) into pSG2 and pSC11 between restriction sites Kpn 1 and Not 1, and confirmed on agarose gel electrophoresis. The correct bands were identified, extracted and used to transform competent bacteria (XL-1 Blue competent cells, Stratagene, UK) grown in selecting antibiotic (kanamycin 50μg/ml for pSG2, ampicillin 100μg/ml for pSC11). PCR based screening of transformed colonies was used by inoculating bacteria into a reaction mix (forward/reverse primers, dNTP,

MgCl<sub>2</sub>, reaction buffer and Taq polymerase (Bioline, London, UK). PCR products were detected on agarose gel electrophoresis. Mini/ Maxi preps of pSG2/CA9 and pSC11/CA9 from transformed colonies were performed as per instructions (Qiagen, London, UK). **pSC11/CA9<sub>254-262</sub>** recombinant plasmid was generated initially by direct insertion of annealed oligonucleotides into pSC11 restriction sites Bgl II and Not I, and expanded along similar lines.

**- Recombinant vaccinia encoding CA9 and CA9<sub>254-262</sub> minigene** were synthesised by homologous recombination of DNA into the thymidine kinase (TK) gene of WR vaccinia virus, using shuttle vector pSC11 as previously described (Chakrabarti et al., 1985). The CA9 segment from pSC5C was cloned into pSC11, as was the CA9<sub>254-262</sub> minigene construct as described. A subconfluent layer of TK143 cells was first infected with  $5 \times 10^5$  plaque forming units (pfu) of WR vaccinia at 37°C for 2 hours. DNA-liposome mixture (20µg DNA + 20µl ddH<sub>2</sub>O + 80µl Opti-MEM + 50µl Lipofectamine; Gibco, UK) was then added to the monolayer after draining off the virus. Thirty minutes later, the cells were drained, washed and cultured in D10 (DMEM media + 10% FCS; Sigma, Dorset, UK) for 2 days. Cultures were assessed for cytopathic effects, and virus harvested by repeated freeze-thaw cycles and collection of the supernatant following centrifugation. Selection of recombinant virus was achieved through BrdU selection of re-infected TK143 cells. Since TK143 cells are TK deficient, wild type WR virus carrying a functional TK gene will be killed by BrdU, thus enriching for recombinant viruses whose TK gene was disrupted by the DNA insert. Following 2-3 days of BrdU selection, recombinant plaques were visualised with an Xgal containing overlay. Since the LacZ

gene is carried by the pSC11 vector, successful recombination should render plaques blue, allowing for physical separation of recombinant viruses. The BrdU selection/ Xgal plaque visualisation process was repeated 2-3 times. Finally, the stock of recombinant virus was further expanded and viral titres in plaque forming units (pfu) determined using a titration assay.

All recombinant plasmids and viruses were sequenced for the correct CA9 and CA9<sub>254-262</sub> minigene inserts (Department of Biochemistry, University of Oxford). In addition, expression of CA9 in pSG2/CA9 and rVac/CA9 transduced MC57 cells were assessed through Western blotting with CA9 specific antibody M75 (gift from Dr Pastorek) as shown in fig 4.2

- **DNA-mel3** Recombinant plasmid containing the mel3 polyepitope construct was provided by Dr MJ Palmowski.
- **pGL3PGKX3** Recombinant plasmid containing murine hypoxia inducible factor (HIF) derived from phosphoglycerate kinase (PGK) and **pcDNA1/Neo/HIF-1 $\alpha$**  containing the HIF-1 $\alpha$  sequence from pBluescript/HIF-1 $\alpha$  3.2-2T7 cloned into pcDNA1, were gifts from Dr Peter Ratcliffe, Wellcome Trust, Oxford

- **rVac/mel3 and rVac/ NY-ESO** Recombinant vaccinia virus encoding the mel3 polyepitope and NY-ESO protein were provided by Dr MJ Palmowski

## **2.5 Drugs**

- **Exochelin** Desferri-exochelin 772SM and iron-loaded exochelin (ferri-exochelin 772SM) were a gift from Keystone Biomedical Inc, Los Angeles CA, USA. The desferri-exochelin 772SM was dissolved in 0.09% saline, sonicated in a water bath and heated to 90°C, giving a stock solution of 0.5mg/ml contained in an iron-free plastic tube. The stock solution was stored at 4°C. Similar preparations were made of ferri-exochelin. During experiments, iron-free minimal essential media was used during the exposure of cell samples to desferri-exochelin, to maximise the intracellular iron chelating effects.

- **Cyclophosphamide** CTX (ASTA Medica Ltd, Cambridge, UK) powder was reconstituted in sterile distilled H<sub>2</sub>O and used within 48 hours as per instructions.

## **2.6 Mice**

- **C57BL/6** mice were from breeding pairs originally obtained from Jackson Laboratories, Bar Harbor, Maine. All mice were maintained at the Biomedical Services Unit of John Radcliffe Hospital by brother x sister mating; in vivo experimental protocols were performed according to institutional guidelines.

- **HHD** transgenic mice expressing chimeric MHC I complex comprising HLA-A2 derived  $\alpha 1$  and  $\alpha 2$  domains with H-2D<sup>b</sup>  $\alpha 3$  domain and human  $\beta 2m$  (Pascolo et al., 1997) were a gift from Dr F Lemmonier (Institut Pasteur, Paris, France) and maintained at the Biomedical Services Unit of John Radcliffe Hospital by brother x sister mating; in vivo experimental protocols were performed according to institutional guidelines.

## IN-VITRO METHODS

### 2.7 In-vitro expansion of lymphocytes

- **In vitro expansion of murine splenocytes.** Spleens were harvested in RPMI, mashed and homogenised through a cell strainer. Red blood cell lysis buffer was added to the cell pellet for 5 minutes and washed. Resulting cell population was divided equally into two halves, with one half pulsed with relevant peptide (1 $\mu$ M) for 1 hour at 37°C and irradiated for 12 minutes at 3.15 gray (caesium radioactive source). Free peptide was washed off and the two populations recombined in R10 media. Cells were plated in 24 well plates at density of 2-4 x 10<sup>6</sup> cells/ well. Murine IL2 (Peprotech, London, UK) at 100u/ml added to media, with fresh media and IL2 replaced every 3 days. Restimulation was done weekly with irradiated syngeneic splenocytes pulsed with 1 $\mu$ M peptide.

- **In vitro priming of healthy HLA-A2 donor.** PBMC from healthy donors were separated by magnetic bead sorting (MACS beads, Miltenyi Biotech, UK) into CD14+

and CD14<sup>+</sup> fractions. The CD14<sup>+</sup> cells were cultured in R10 media in the presence of IL-4 and GM-CSF to generate DC which were subsequently matured with CD40L transfected J558 cells (confirmed by FACS analysis of expression of maturation markers CD80 and CD86), and loaded with CA9<sub>254-262</sub> peptide (10 uM) before priming autologous CD14<sup>+</sup> - PBL. The PBL were cultured in IL2 (Proleukin. Chiron, Emeryville, CA, USA) supplemented hR5 media comprising RPMI with 5% pooled human sera, 1% non-essential amino acids, 1% sodium pyruvate, 2 mM glutamine, 1 % penicillin-streptomycin, 5 x 10<sup>-5</sup> M 2-mercapto-ethanol (Invitrogen Ltd, Paisley, UK), with fresh media and IL2 exchanged every 3-4 days. On day 11 post priming, PBL were restimulated with irradiated allogeneic CD14<sup>+</sup> cells that had been pulsed with CA9<sub>254-262</sub> peptide (1 uM). On day 10 following the last restimulation, cells were stained with HLA-A2/CA9<sub>254-262</sub> tetramers and anti CD8 antibodies, and analysed by FACS.

**- In vitro expansion of CA9 specific PBL from HLA-A2 RCC patients.** PBMC from RCC patients were thawed and individually restimulated with relevant peptide (100µM for CA9 peptides, 10µM for control pooled peptides) in 100µl hR5 media at 37°C for 1 hour.

Individual CA9 peptides comprised:

- 1) CA9<sub>26-35</sub> LLSLLLLMPV
- 2) CA9<sub>254-262</sub> HLSTAFARV
- 3) CA9<sub>271-279</sub> GLAVLAAFL
- 4) CA9<sub>421-429</sub> GLLFAVTSV

Control peptides consisted of pooled peptides from three well characterised viral epitopes derived from influenza, Epstein-Barr and cytomegalovirus:

- 1) Flu : GILGFVFTL
- 2) EBV : GLCTLVAML
- 3) CMV : NLVPMVATV

Following restimulation, cells were plated in hR5 supplemented with IL7 (25ng/ml. Peprotech, London, UK)) without washing off peptide in 96 well plates at  $5-10 \times 10^5$  cells/well, with each peptide restimulation plated in duplicate. On day 3 following initial restimulation, IL2 at 100U/ml was added to fresh media. Media with IL2 was replaced every 3-4 days. Restimulation was repeated every 10-12 days with 10 $\mu$ M peptide pulsed irradiated .221A2 LCL at a ratio of 3:1 (feeders : responders).

- **FACSorting.** CD8+/ CA9<sub>254-262</sub> and CA9<sub>26-35</sub> tetramer<sup>hi</sup> CTLs were isolated from PBL by FACSorting, which was performed at the sorting facility at the Weatherall Institute of Molecular Medicine, Oxford. Cells were first stained with CD8 antibodies and PE-labelled tetramers just prior to sorting. Tetramer<sup>hi</sup> cells were sorted at one cell per well or 5-50 cells / well into 96 well plates. The sorted cells were restimulated with irradiated allogeneic PBL and B cell feeders in hR5 media containing IL-2 (100U/ml) and phytohemagglutinin PHA.

## **2.8 Phenotype and functional analyses of CTL**



- **Tetramer and FACS analysis.** For tetramer analysis of specific murine and human CTL, cells were initially incubated with relevant tetramer at 37°C for 20 minutes, washed and stained with corresponding anti CD8 antibody on ice for 10 minutes (FITC conjugated mouse anti-human CD8; FITC conjugated rat anti-mouse CD8. BD Pharmingen, San Diego, CA, USA). Prior to acquisition by FACS, samples were stained with 0.5 µg propidium iodide to gate out dead cell debris. Cells were analysed with FACScan hardware and CellQuest software (BD Biosciences, Mountain View, CA).

- **Interferon  $\gamma$  (IFN $\gamma$ ) ELIspot assay.** ELIspot plates (multiscreen-IP plates, Millipore, UK) were prepared according to manufacturers recommendations (Mabtech AB IFN $\gamma$  kit). Responder cells were plated at  $1 \times 10^5$  cells/well and stimulated with 10µM peptide in R10 media for 16 hours. Plates were developed and read on an ELIspot Reader System (Autoimmun Diagnostika, Strassberg). For recall assays on PBL from RCC patients, responder cells were directly transferred from 96 well plates onto the ELIspot plate without prior enumeration. Equal sample volumes (and cell numbers) were transferred into corresponding peptide pulsed, negative control (unpulsed) and PHA stimulation wells.

-  **$^{51}$  Chromium release cytotoxicity assay.** Targets were resuspended in 50µl fetal calf serum and incubated with 50-150µl of  $^{51}$ Cr (sodium chromate solution, Amersham Pharmacia Biotech, Little Chalfont, UK) for 90 minutes at 37°C. Cells were washed in RPMI and pulsed with peptide for 1 hour at 37°C or else left unpulsed. Following the removal of free peptide, cells were transferred into 96 well plates and co-incubated with



CTL effectors in R10 media at various effector : target ratios (E:T ratio). Spontaneous  $^{51}\text{Cr}$  release was accounted for by incubation of  $^{51}\text{Cr}$  loaded targets alone, whilst maximum release was achieved with 10% Triton solution. After 5 hours incubation at  $37^\circ\text{C}$ ,  $50\mu\text{l}$  of supernatant was mixed thoroughly with  $150\mu\text{l}$  scintillant (Microscint, Packard, UK) and  $\gamma$  radioactivity measured with a  $\gamma$  counter Topcount.NXT (Packard, UK). Specific lysis of targets was calculated as follows:

$$(\text{sample} - \text{spontaneous release} / \text{maximum} - \text{spontaneous release}) \%$$

**- Viral transduction of target cells.** For all assays using virally transduced targets, cells were infected at multiplicity of infection (MOI) of 10:1, in virus dilution media (RPMI + 0.1% bovine serum albumin) at  $37^\circ\text{C}$  for 2 hours. After washing off the virus dilution media with RPMI, cells were resuspended in R10 for 16 hours at  $37^\circ\text{C}$  to allow for expression of the viral construct.

## **2.9 Other molecular biological techniques**

**-  $\beta$ galactosidase assay.**  $\beta$ gal activity in Z16 cells was assessed with  $\beta$ gal assay system (Promega, Madison, WI, USA). Cells were lysed in-situ with lysis buffer and harvested. Samples were vortexed and stored at  $-70^\circ\text{C}$ . For analysis, thawed lysates were diluted with reporter lysis and plated in a 96 well plate. Assay buffer was added and incubated for 30 minutes at  $37^\circ\text{C}$ . The reaction was stopped by adding 1M Sodium Carbonate, and colour reaction read in a plate reader at 420nm. Background controls were established with assay buffer alone without lysate.

- **HuDMEC proliferation assay.** Cell counts on HuDMEC at various time points were performed in a Coulter counter (Beckman Coulter, High Wycombe, UK) after trypsinization and resuspension in buffered solution. Cells were grown in 6 well plates, media pulsed with VEGF (8ng/ml) with or without desferri-exochelin 10 uM on days 0, 2 and 4.

- **Normoxic/Hypoxic incubation.** Cells were exposed to normoxic conditions (Forma Scientific CO<sub>2</sub> water jacketed incubator) at 37°C, 5%CO<sub>2</sub> and 21% oxygen. Hypoxic conditions were maintained for 16 hours at 37°C, 5%CO<sub>2</sub> and 0.1-1.0% oxygen in nitrogen-flushed hypoxic chambers (Heto Cellhouse 170 HI).

- **Western blotting.** Cells were lysed in 8M urea lysis buffer, and homogenized lysates were standardized for protein levels (BioRad protein assay kit, Bio-Rad laboratories, Hercules, CA) before separation on 6% SDS polyacrylamide gels and transferred onto Immobilon membranes (Millipore, Bedford, MA) with semidry blotter Imm-2 (W.E.P. co., Concord, CA). Membranes were blotted with primary antibodies as appropriate. Secondary horse radish peroxidase conjugated goat antimouse antibody (DAKO Denmark) was applied at 1:1000 and chemiluminescence detected with ECL Western blotting kit (Amersham, Buckinghamshire, UK). After analysis, all membranes were stained with Ponceau S (Sigma, Poole, UK) to verify equal protein loading and transfer. Bands were quantified by spot densitometry using normoxia as baseline (ImageQuant software, Molecular Dynamics, Amersham Pharmacia Biotech, UK).

Primary antibodies used:

- mouse anti-human HIF 1 $\alpha$  antibody 1:1000 (Transduction Laboratories, Becton Dickinson UK, Oxford, UK)
- anti-NIP3 antibody 1:100 (a gift from Arnold H Greenberg, the Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Canada)
- M75 monoclonal antibody to human CA9 1:50 (gift from Dr Pastorek, Slovak Academy of Sciences, Slovak Republic)

- **VEGF measurements in culture media.** VEGF levels were assayed using the Quantikine Human VEGF ELISA kit (R&D systems, Minneapolis, MN). Samples were obtained from cell culture supernatants at the time of cell harvesting for protein lysates. The assays were performed in quadruplicate, and read on a plate reader ( $\mu$ Quant. Bio-Tek, Winooski, Vermont, USA) at 540nm.

- **RNase protection assays for VEGF transcripts.** RNA was extracted from cultured cells using Tri reagent (Sigma, Dorset, UK). Quantification of RNA was performed spectrophotometrically at 260/280 nm wavelength. Antisense VEGF and loading control (U6 small nuclear RNA) riboprobes were prepared from cDNA constructs (VEGF<sub>121</sub> accession no.M95200, U6 accession no.X01366) using cytosine triphosphate (CTP) labelled with <sup>32</sup>P. Twenty ug of total RNA samples were hybridised to the riboprobes overnight. RNase digest was performed the next day with 20ul RNaseT1 and 20ul RNaseA for 40 minutes. Samples were precipitated, resuspended in loading buffer and resolved for 2 hours by electrophoresis in a polyacrylamide gel (Sequa gel, National

Diagnostics) . The gel was then exposed to Xray film at room temperature and quantified by spot densitometry (ImageQuant software, Molecular Dynamics, Amersham Pharmacia Biotech, UK).

**- Luciferase reporter assays.** The pGL3 plasmid (Promega, Southampton, UK) contained the luciferase gene linked to the SV40 promoter. Three consecutive repeats of 12 bp mouse phosphoglycerate kinase 1 HRE were linked to pGL3, to generate pGL3PGKX3 (gift from Dr Ratcliffe, Wellcome Trust, Oxford, UK). Both C4.5 and Ka13 cells were transfected with pGL3PGKX3 using FuGENE6 (Roche Diagnostics, East Sussex, UK) transfection reagent. Controls with a SV40 promoter that lacked a HRE. All cells were plated at  $7 \times 10^5$  cells/well and transfected with HRE or non-HRE linked pGL3 plasmid (and cotransfected with lacZ- $\beta$  galactosidase plasmid). Some Ka13 cells were cotransfected with a human HIF-1 $\alpha$  cDNA plasmid (pcDNA1/Neo/HIF-1 $\alpha$  containing the HIF-1 $\alpha$  sequence from pBluescript/HIF-1 $\alpha$  3.2-2T7 cloned into pcDNA1). Samples treated with 10  $\mu$ M desferri-exochelin 772SM for 16 hours. Luciferase detection with the Luciferase Assay System (Promega , Southampton, UK), and normalised to  $\beta$  galactosidase activity.

**- Cell cycle analysis using propidium iodide/ FACS.** Equal numbers of subconfluent MDA468 tumour cells that were previously treated with desferri-exochelin (1 and 10  $\mu$ M) were harvested in trypsin/EDTA and washed in PBS. Following centrifugation, supernatant was discarded and cell pellets fixed in 2ml cold 70% ethanol and vortexed to prevent clumping. Cells were left on ice for 30 mins, and then washed with PBS and

centrifuged. Supernatant was discarded and cell pellets stained in 1ml PBS with 40µg/ml propidium iodide and 100µg/ml RNase A, and left in the dark at room temperature for 15 mins before analysis by FACS in FL-2 channel.

## **2.10 Immunohistochemistry**

- **Staining of βgal in Z16 tumour sections.** Tumours were harvested in formalin, paraffin embedded, sectioned and mounted on glass slides. The rabbit Envision kit (DAKO, Denmark ) was used as per instructions following dewaxing and rehydration. Primary rabbit anti-βgal antibody was employed, with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody. Sections were developed with DAB and chromogen and counterstained with haematoxylin.

- **Staining of human CA9 in RCC sections.** Mounted tumour sections were obtained from the Department of Histopathology, John Radcliffe Hospital. DAKO Envision kit HRP/DAB (DAKO, Denmark) was used as per instructions following dewaxing and rehydration. M75 anti-human CA9 primary antibody was used, with HRP-conjugated goat anti-mouse secondary antibody. Sections were developed with DAB and chromogen and counterstained with haematoxylin. Slides were reviewed with the expert assistance of Dr D Davies.

## **IN-VIVO METHODS**

### **2.11 (Chapter 3) : Immunisation against tumour antigen βgal in C57BL/6 mice**

- **Immunisation of C57BL/6 mice against tumour antigen  $\beta$ gal.** To generate specific T cell responses against tumour antigen  $\beta$ gal, one  $\mu$ g of  $\alpha$ -galactosylceramide ( $\alpha$  GalCer; provided by Kirin Breweries, Japan) and 400  $\mu$ g of  $\beta$ galactosidase ( $\beta$ gal; Calbiochem, UK) protein dissolved in sterile PBS were administered intravenously into each mouse. Seven days later, the mice were boosted with recombinant vaccinia encoding for full length  $\beta$ gal, by intravenous injection of  $1 \times 10^6$  pfu / 200 $\mu$ l of sterile PBS per mouse.

- **Monitoring *ex vivo* responses to  $\beta$ gal in immunised C57BL/6 mice.** Immunised mice were tail-bled as per standard procedure, and blood specimens lysed immediately with lysis buffer at room temperature for 30 minutes. Separation of PBL pellet was achieved by centrifugation at 4000 rpm for 4 minutes, and supernatant siphoned off. PBL were stained and analysed by FACS as described.

- ***In vivo* tumour challenge assays.** Mice were challenged with tumour administered subcutaneously into the flanks or intravenously into the tail vein, with tumour load varying between  $2 - 5 \times 10^5$  cells. In the subcutaneous model, measurements were taken twice a week, and tumour size for each group was calculated as the mean of the products of bisecting diameters ( $\pm$  SD). Measurements were terminated for each group when the first animal developed a tumour in excess of 200 mm<sup>2</sup>. In the intravenous model, all mice were sacrificed at a predetermined day post challenge, and measurements expressed as dry lung weights.

## **2.12 (Chapter 4) : Immunisation against human CA9<sub>254-262</sub> epitope in HHD mice**

- **DNA + recombinant vaccinia (prime/ boost).** DNA priming with full length CA9 (pSG2/CA9) was performed intramuscularly in each anterior quadricep with 50 $\mu$ l (40 $\mu$ g DNA) solution. Twenty one days later, mice were boosted with recombinant virus (rVac/CA9) intravenously with  $1 \times 10^6$  pfu per mouse.

- **Peptide pulsed bone marrow derived Dendritic cells (BMDC).** Bone marrow was harvested and cellular extract cultured in R10 media supplemented with GM-CSF (10ng/ml) and IL4 (1ng/ml) (Peprotech, London, UK). On day 7, cells were pulsed with 10 $\mu$ M peptide and injected subcutaneously at  $5 \times 10^5$  cells in each flank.

- **Peptide + Incomplete Freund's adjuvant.** A 100 $\mu$ g of peptide was mixed in 1:1 volume ratio with incomplete Freund's adjuvant (Sigma, Dorset, UK), sonicated, vortexed and injected subcutaneously into multiple sites on the flanks and back.

### **2.13 (Chapter 6) : Immunisation against the mel3 epitope in C57BL/6 mice**

- **Immunization strategies.** Animals were primed for influenza NP<sub>366-374</sub>-specific CTL responses by intramuscular injection of 50  $\mu$ g of plasmid DNA encoding the *mel3* polyepitope (DNA-mel3). The mel3 construct (Choi et al., 2002) consists of a string of five HLA-A2 and two HLA-A1 melanoma epitopes, and the influenza nucleoprotein epitope restricted by H-2D<sup>b</sup> (ASNENMDAM). Only the NP<sub>366-374</sub> epitope is presented on a C57BL/6 background. Mice were boosted 14-30 days after DNA immunization by i.v. injection with  $10^6$  PFU of recombinant MVA encoding the *mel3* polyepitope construct (MVA-mel3). In some instances, animals immunized against mel3 were boosted a second



time by i.v injection with  $1 \times 10^6$  syngeneic splenocytes that had been infected with recombinant vaccinia encoding the *mel3* polyepitope construct (Vacc-mel3). Splenocytes ( $5 \times 10^7$  cells/ml) were infected with  $10^8$  PFU of Vacc-mel3 in PBS supplemented with 0.1 % BSA (Sigma-Aldrich) at 37°C for 2 h, washed, and injected i.v. into the lateral tail vein. To generate LCMV GP<sub>33-41</sub> specific CTL, mice were immunised with sc injection of  $5 \times 10^5$  syngeneic bone marrow derived dendritic cells (BMDC) pulsed with 10 µM LCMV GP<sub>33-41</sub> peptide (KAVYNFATM) for 2 hours in complete media.

**- Monitoring murine CTL responses with MHC tetramers.** Tetrameric H-2 D<sup>b</sup>/NP<sub>366-374</sub> peptide complexes were prepared as outlined in Altman *et al.* (Altman et al., 1996), and used to stain fresh PBL isolated from the lateral tail vein. Approximately  $5 \times 10^5$  PBL were suspended in 20 µl CM and incubated with 0.5 µg of tetramer complexes at 37°C for 20 min. The cells were then incubated with anti-CD8α and anti-CD43 (BD Pharmingen, San Diego, CA) for 10 min at 4°C, washed twice with PBS, and resuspended in PBS for FACS analysis. Blood was also collected to perform white blood cell counts so that total lymphocyte numbers per mm<sup>3</sup> of blood could be extrapolated. White blood cell counts were performed using a Micros60 blood counter (ABX-PARC Euromedecine, France).

**- Cyclophosphamide dosing schedule.** CTX (ASTA Medica Ltd, Cambridge, UK) was reconstituted in sterile distilled H<sub>2</sub>O and administered by intraperitoneal injection. The dosing schedules of Browder *et al.* (Browder et al., 2000) were adopted for these studies. Thus metronomic dosing consisted of 175 mg/kg of CTX injected every 6 days, and



standard dosing consisted of a 21 day cycle of 150 mg/kg CTX administered every other day over 6 days (ie. three injections) followed by 15 days rest.

- **Tumor immunity assay.** One week after immunotherapy, groups of mice ( $n = 5$ ) were challenged with B16.F10 melanoma cells (C57BL/6, H-2<sup>b</sup>) that had been modified to express the *mel3* polyepitope construct in combination with enhanced green fluorescent protein from a bicistronic mRNA (pIRES2-EGFP vector, Clontech, Basingstoke, UK) (Prevost-Blondel et al., 1998). Challenge was with  $3 \times 10^5$  tumor cells injected s.c. into the left flank. Mice were monitored for tumor growth every 3-4 days, and tumor size for each group was calculated as the mean of the products of bisecting diameters ( $\pm$  SE.). Measurements were terminated for each group when the first animal developed a tumor in excess of 200 mm<sup>2</sup>.

## STATISTICAL ANALYSES

Statistical analysis was done on PRISM statistical software (GraphPad software, San Diego, USA) . For comparison of two means (unmatched), the two-tailed independent Student's T test was employed at 95% level of confidence. Findings were regarded as significant if two-tailed  $p$  values were  $< 0.05$ . For comparison of multiple means (unmatched), analysis of variance (ANOVA) with multiple comparisons testing was used at 95% confidence limits,  $p < 0.05$  significance level. For categorical data, the chi-square test was employed at 95% confidence limits,  $p < 0.05$  significance level.

## Chapter 3. A hypoxia-regulated antigen as a T cell target in a mouse model

### 3.1 Introduction

In this chapter, the hypothesis was that a hypoxia regulated tumour antigen expressed under hypoxic conditions, would be sufficient to induce specific T cell responses. It was also assessed whether hypoxia inducible expression of a tumour antigen could have anti-tumour effects in vivo.

The effect of hypoxia on the innate immune response has been described. Turner (Turner et al., 1999) reported that hypoxia played a role in macrophage accumulation in necrotic areas through inhibition of chemotactic factor monocyte chemoattractant protein (MCP-1), and this was confirmed by Bosco (Bosco et al., 2004). Schioppa (Schioppa et al., 2003), reported that low oxygen tension induced high expression of chemokine receptor CXCR4 in monocytes and macrophages through HIF-1 $\alpha$ , which was paralleled by an increased chemotactic responsiveness to its specific ligand stromal-derived factor 1 (SDF-1 or CXCL12). Cramer (Cramer et al., 2003) elegantly showed in mice that a conditional knockout of HIF-1 $\alpha$  in macrophages and other myeloid lineage cells led to profound impairment of the inflammatory responses involving these cells. It was indeed a striking demonstration that a single molecule such as HIF-1 $\alpha$  could play so pivotal a role in two seemingly diverse and significant settings as hypoxia and inflammation. Blouin *et al* (Blouin et al., 2004) similarly showed that lipopolysaccharide (LPS) induced hypoxic gene activation in macrophages was blocked by a dominant negative form of HIF-1 $\alpha$ .

Therefore hypoxia together with non-hypoxia driven factors like LPS, play an essential role in activating the innate immune response through the common denominator of the HIF-1 $\alpha$  transcription factor. On the other hand, Sitkovsky (Sitkovsky, 2004) suggested that hypoxia also acted as a immune regulator of the inflammatory response, through accumulation of extracellular adenosine and activation of immunosuppressive A<sub>2 $\alpha$</sub>  adenosine receptors in activated cells. Besides monocytes/ macrophages, Fink (Fink et al., 2003) reported that NK mediated cytotoxicity was significantly inhibited in hypoxia, which was partly reversed by IFN $\alpha$ . Overall therefore, it would seem that hypoxia plays a dual homeostatic role in both activation and suppression of the innate immune response; activation to allow initial containment and eradication of pathogens followed by suppression of the immune response to prevent excessive collateral damage.

With regard to the adaptive immune response, Loeffler (Loeffler et al., 1992) showed that IL-2 induced lymphocyte proliferation was reduced in hypoxia, as did Naldini (Naldini and Carraro, 1999) with mitogen-activated PBMC, the latter being attributed to the decreased synthesis of mitotic cyclins A and B. Conforti (Conforti et al., 2003) reported that hypoxic inhibition of voltage-dependent potassium channels in T lymphocytes accounted for inhibition of proliferation induced by T cell receptor (TCR) cross-linkage. Loeffler (Loeffler et al., 1990) also showed that, unlike monocyte/ macrophage migration, lymphocyte migration into EMT6 mouse mammary tumours appeared to infiltrate all areas of oxygenation *in vivo* equally well. This was also later confirmed by Turner (Turner et al., 1999) who demonstrated that hypoxia did not affect MCP-1 induced migration of lymphocytes. An interesting publication by Caldwell (Caldwell et

al., 2001) showed that although CD8 T lymphocyte proliferation was affected more than CD4 cells in hypoxia, the overall lethal hit delivery was maintained, suggesting that the fewer remaining CD8 cells in hypoxia achieved a higher lytic activity on a per cell basis compared with normoxia. This was attributed to a much higher proportion of TCR<sup>HI</sup> and LFA<sup>HI</sup> cells among the CD8 T lymphocytes, resulting in higher lytic activity. IFN $\gamma$  and IL2 production was still present in hypoxically incubated TCR activated CTL, albeit at lower levels compared with normoxia, this again being due to reduced overall cell numbers in hypoxia. Overall, it would seem that hypoxia inhibits lymphocyte proliferation without significantly affecting function. With regard to T cell apoptosis and hypoxia, adrenomedullin was reported to protect activated T cells from antigen induced cell death (AICD) following induction by both hypoxia and TCR activation through HIF-1 $\alpha$  (Makino et al., 2003). BNIP3, a member of the hypoxia regulated Bcl-2 pro-apoptotic family (Bruick, 2000), was found to induce AICD of effector CTL in a caspase-independent fashion (Wan et al., 2003). These studies suggest that the overall survival or apoptotic signals in activated T cells depend on the fine balance between two hypoxia regulated products with opposing functions, a paradigm also manifest in tumour cells (see Chapter 7). Just as in the innate immune response, hypoxia could play the dual role of promoting and then suppressing the adaptive response.

The effects of hypoxia on the immune response to tumours however, is more imbalanced, with the evidence in favour of tumour growth and spread. Tumour associated macrophages have been implicated in tumour progression through hypoxia and cytokine driven HIF-2 $\alpha$  expression and angiogenesis (Bingle et al., 2002; Knowles et al., 2004),

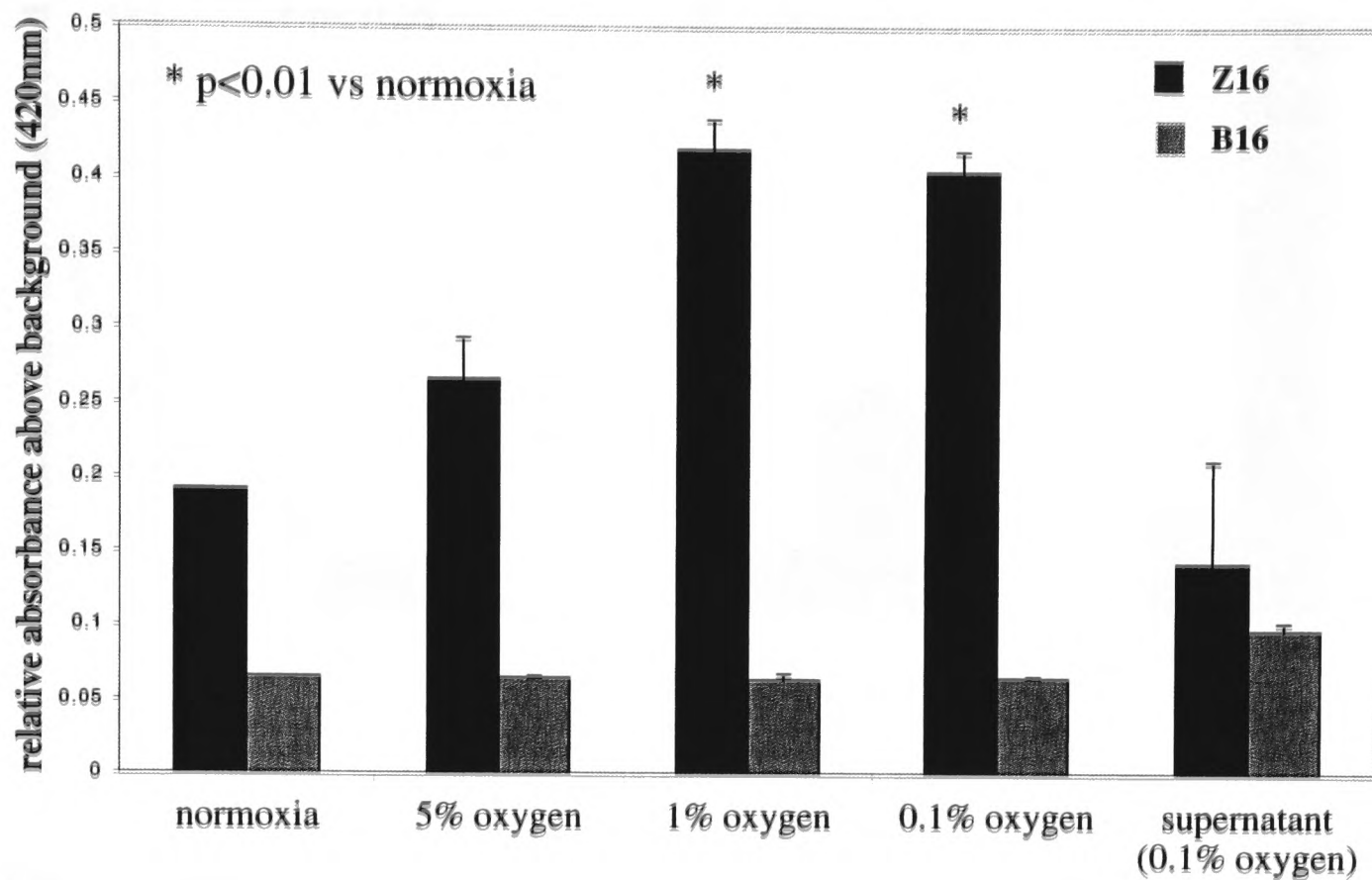
secretion of tumour growth factors as well as suppression of anti-tumour immune responses (al-Sarireh and Eremin, 2000). Hypoxia has been suggested to skew the adaptive response to a Th2 phenotype which has been associated with increased cancer risk (Joon Yun et al., 2004). To date, there is a dearth of evidence on the effects of hypoxia on the adaptive immune responses to tumours; whether tumours are rendered more or less immunogenic, and whether hypoxia-regulated tumour antigens could prove useful as immune targets. It is known that responses to cellular stresses such as heat shock, hyperoxia and hypoxia, result in induction of protein expression (Moseley, 2000). However, it is still unclear whether up-regulation of stress-inducible proteins related to tumour hypoxia can result in altered processing and presentation of tumour antigens, perhaps contributing to tumour escape by presenting novel epitopes that are not recognised by activated T cells.

To study the effects of specific anti-tumour T cell immunity against a hypoxia-regulated tumour antigen, a clonal murine tumour cell line Z16 (gift from Prof Ian Stratford, Dept of Pharmacology, University of Manchester) that contains the hypoxia responsive reporter construct  $\beta$  galactosidase ( $\beta$ gal) was used. The  $\beta$ gal gene was inserted downstream of a SV40 promoter, and driven by the hypoxia responsive murine phosphoglycerate kinase (PGK) promoter. The construct was stably transfected into the wild type murine melanoma tumour cell line B16F10, and positive cells enriched by neomycin selection. To directly study the subpopulation of CTL that recognise a specific peptide/MHC complex, the use of tetramer based FACS analysis was employed. Tetramers are multimers of peptide/ MHC complexes linked to a fluorochrome-

streptavidin core. A novel tetramer consisting of a published  $\beta$ gal H-2 K<sup>b</sup> restricted peptide  $\beta$ gal<sub>96-103</sub> DAPIYTNV (Overwijk et al., 1997) was generated to monitor  $\beta$ gal specific CD8 + T cell responses. For induction of  $\beta$ gal specific T cell responses *in vivo*, C57BL/6 mice were vaccinated with  $\beta$ gal protein and CD1d-binding glycolipid  $\alpha$ -galactosylceramide ( $\alpha$  GalCer) as described in Materials and Methods.  $\alpha$  GalCer adjuvant activates natural killer T cells (NKT) resulting in the maturation of dendritic cells (DC) (Kawano et al., 1997), and has been shown to enhance T cell activation *in vivo* (Hermans et al., 2003).

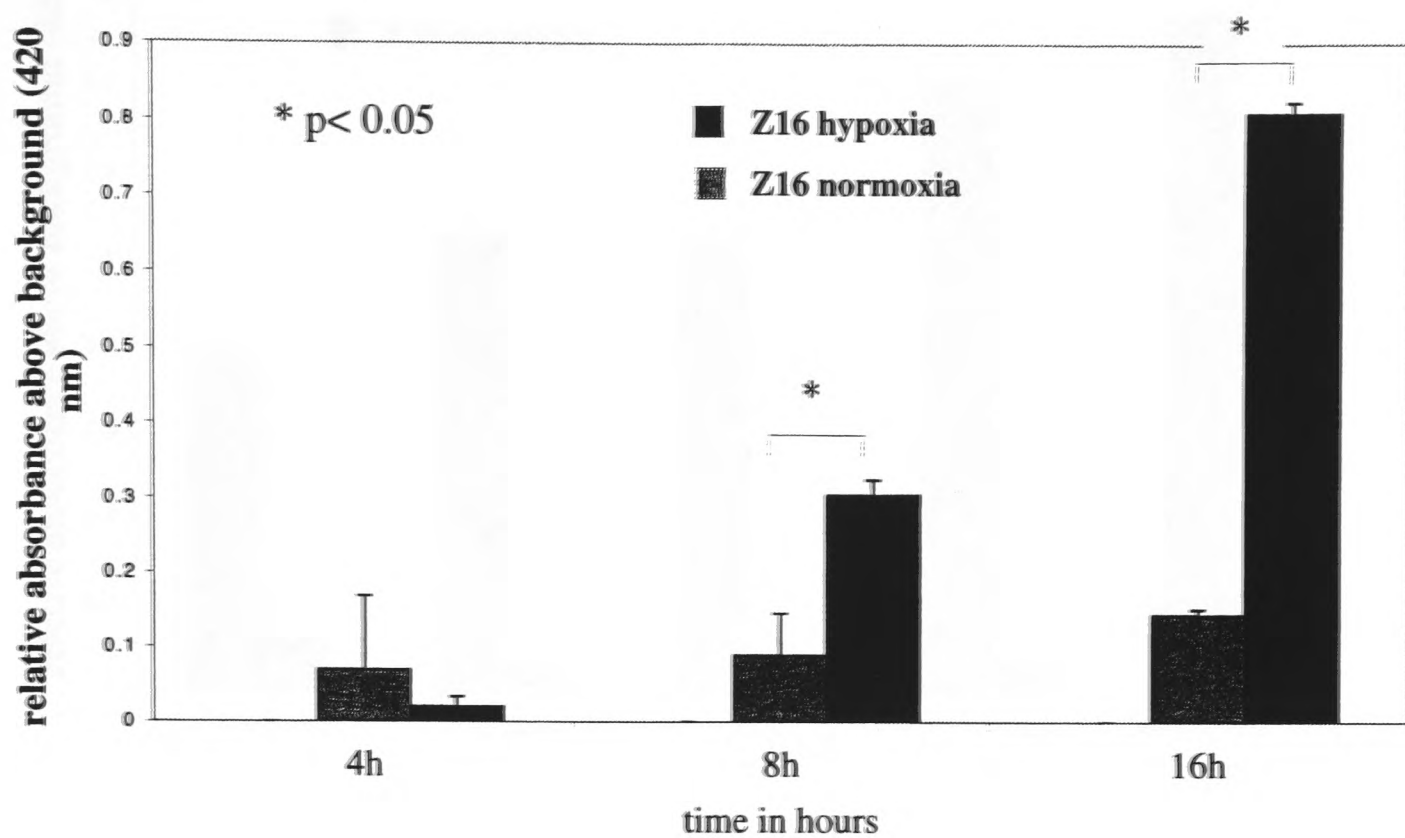
### **3.2 Characterisation of the hypoxia response of a novel $\beta$ -galactosidase ( $\beta$ gal) expressing murine tumour line Z16**

The expression of  $\beta$ gal activity by Z16 under hypoxia was tested under various conditions using a functional  $\beta$ gal assay. Briefly,  $\beta$ gal hydrolyses a colourless substrate into o-nitrophenol that appears yellow and was read spectrophotometrically at 420nm, with absorbance correlating linearly with  $\beta$ gal activity.  $\beta$ gal activity was significantly increased ( $p < 0.01$ ) at 1.0% oxygen over 16 hours compared to normoxic controls, with no further increase at 0.1% oxygen (fig 3.2.1). There was no  $\beta$ gal activity detected in the supernatant over control (untransfected B16), indicating that  $\beta$ gal was confined within the cell. Comparison was made of  $\beta$ gal induction at 1.0% oxygen at varying time points of hypoxic incubation (fig 3.2.2). There was a positive correlation between time in hypoxia and degree of  $\beta$ gal induction, in comparison to normoxic controls ( $p < 0.05$ ). Re-oxygenation of Z16 after hypoxic incubation revealed stability of  $\beta$ gal activity up to 16 hours post re-oxygenation compared with normoxic controls (fig 3.2.3).



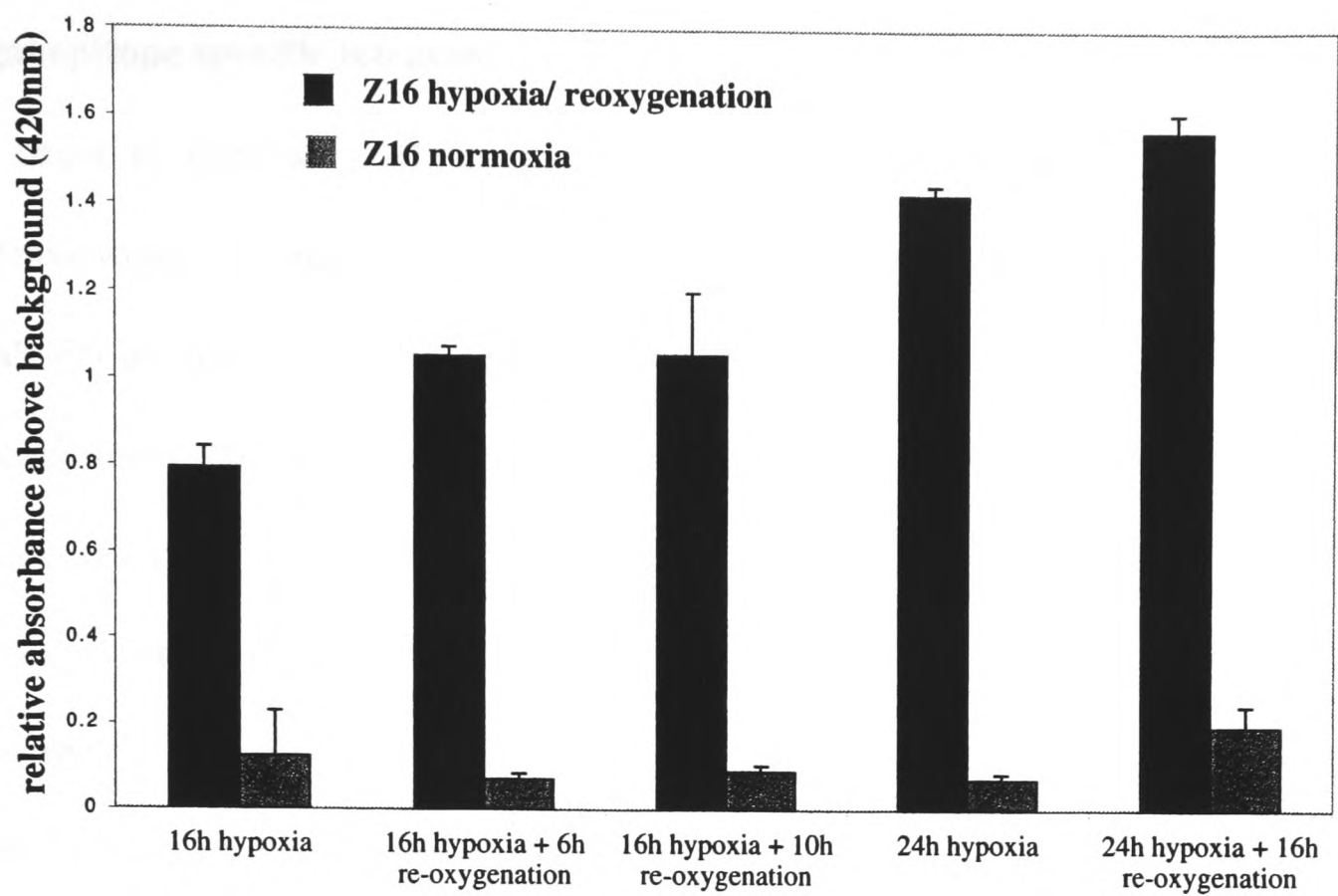
**Fig 3.2.1  $\beta$ gal activity of Z16 varies with oxygen tension.** Hypoxic induction of  $\beta$ gal in Z16 tumour cells was analysed by  $\beta$ gal activity on a substrate that was read spectrophotometrically at 420 nm. Z16 cells were incubated at 37 C over 16 hours at varying oxygen tensions. Levels of  $\beta$ gal induction were compared with wild type B16 controls under similar conditions. Supernatants from Z16 and B16 cells at 0.1% oxygen were assayed for  $\beta$ gal activity. Z16 black bars, B16 gray bars. (*Samples in duplicate, mean values with 1 SD shown. \*Comparison of means using one way ANOVA with Dunnetts multiple comparisons test*)





**Fig 3.2.2 Induction of  $\beta$ gal activity of Z16 varies with duration under hypoxia.** Z16 cells were incubated at 37 C at 1% hypoxia over varying time points. Hypoxic induction of  $\beta$ gal activity was assayed in Fig 3.2.1. Levels of  $\beta$ gal induction were compared with Z16 in normoxia and wild type B16 controls under similar conditions. Z16 (hypoxia) black bars, Z16 (normoxia) gray bars. B16 bars not seen (values < 0.01). (Mean values with 1 SD shown. \*Comparison of means using 2-tailed *t* test)



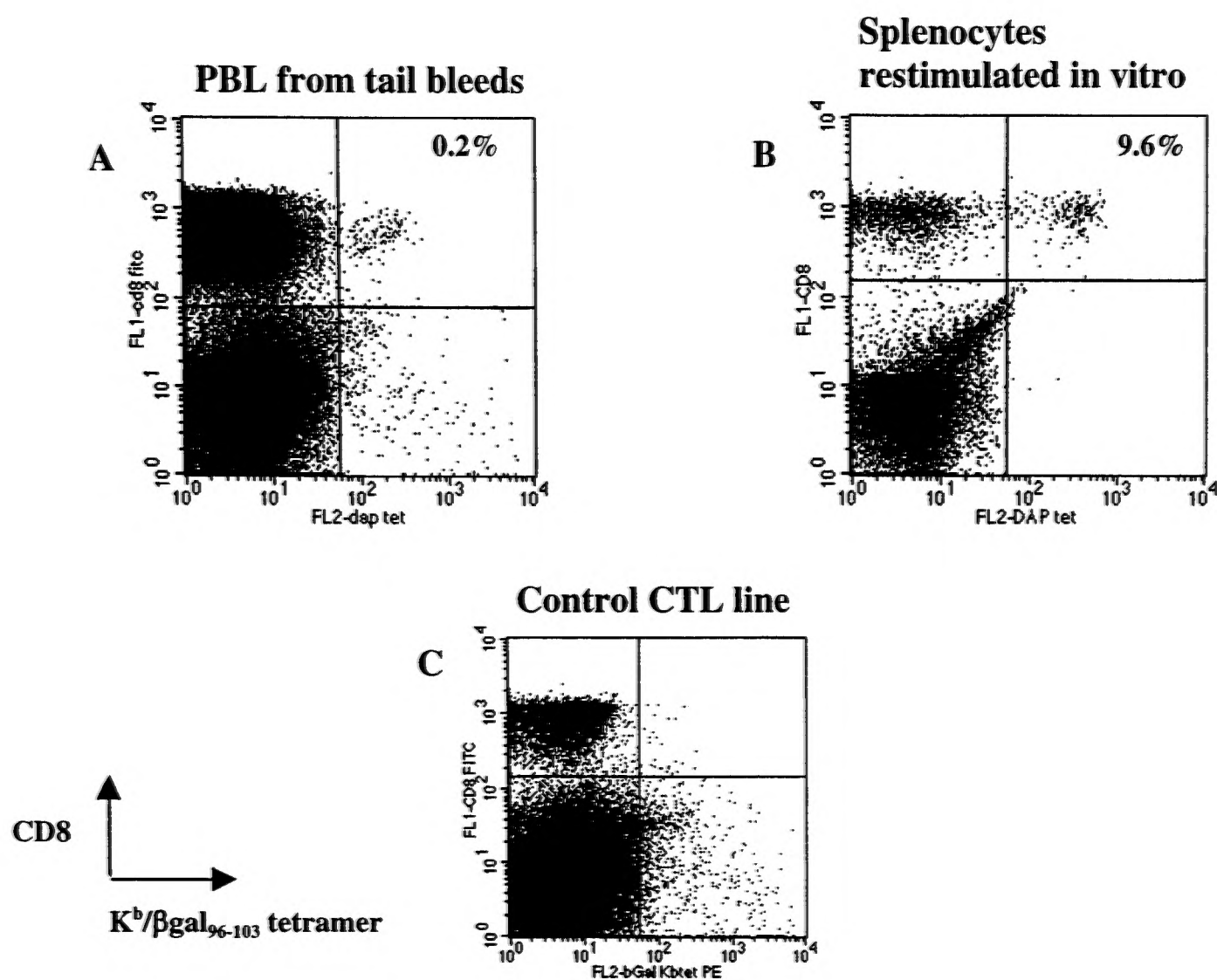


**Fig 3.2.3 Induction of  $\beta$ gal activity in Z16 under hypoxia is stable for up to 16 hours upon re-oxygenation.** Z16 cells were incubated at 37 C in hypoxia (1.0% oxygen tension) and re-oxygenated to 20% oxygen tension at indicated durations, after which  $\beta$ gal activity was assayed . Levels of  $\beta$ gal induction were compared with Z16 in normoxia at similar durations. (Z16 (hypoxia) black bars, Z16 (normoxia) gray bars. Mean values with 1 SD shown)

### 3.3 Generation and monitoring of $\beta$ gal specific CTL responses *in vitro* using a novel $\beta$ gal epitope specific tetramer

In order to generate a  $\beta$ gal specific CTL response *in vivo*, C57BL/6 mice were intravenously primed with  $\beta$ gal protein and the adjuvant  $\alpha$ -galactosylceramide ( $\alpha$  GalCer) as described. The responses were boosted 7 days later with iv injection of recombinant vaccinia virus encoding the full length  $\beta$ gal gene. To monitor the CTL responses generated, H-2K<sup>b</sup>/  $\beta$ gal<sub>96-103</sub> tetramers were used for staining of peripheral blood lymphocytes (PBL) *ex vivo*, followed by acquisition and analysis by flow cytometry. The results in fig 3.3A show a representative FACS plot of PBL from vaccinated mice (tetramer+ / CD8+ population = 0.2%).

To further enrich the  $\beta$ gal specific CTL line, some immunised mice were sacrificed and spleens harvested for further *in vitro* expansion as described. Briefly, this was achieved by *in vitro* restimulation using irradiated  $\beta$ gal<sub>96-103</sub> peptide-pulsed syngeneic splenocytes from naïve mice. Tetramer staining and flow cytometric analysis 7 days later showed enrichment of tetramer+ cells. Fig 3.3B shows a representative FACS plot of *in vitro* restimulated splenocytes (tetramer+ / CD8+ population = 9.6% ), with a negative control for tetramer staining in fig 3.3C. The results demonstrate the specificity of the  $\beta$ gal tetramer, as well as the successful induction of  $\beta$ gal specific CTL responses by *in vivo* priming with  $\beta$ gal protein and adjuvant  $\alpha$  GalCer, followed by recombinant vaccinia boosting.



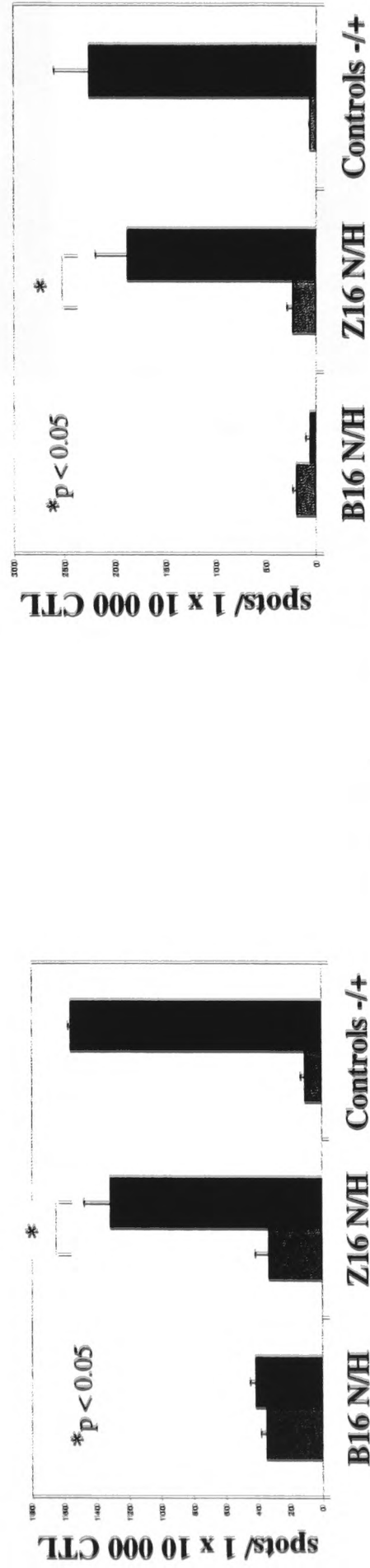
**Fig 3.3 In vitro enrichment of H-2 K<sup>b</sup>/βgal<sub>96-103</sub> specific CTL.** Representative FACS plots of PBL from tail bleeds and splenocytes of C57BL/6 mice. Mice were immunised with βgal protein and α-galactosylceramide adjuvant. Seven days later the animals were boosted with recombinant vaccinia virus encoding the βgal gene. The mice were sacrificed a week later and spleens harvested. Splenocytes were restimulated with βgal<sub>96-103</sub> peptide at 10μM and cultured in vitro with murine IL2. Splenocytes were stained with H-2K<sup>b</sup>/βgal<sub>96-103</sub> tetramer and anti-CD8 antibody, and analysed by flow cytometry. (A) *Ex vivo* analysis from tail-bled PBL before in vitro restimulation of splenocytes. (B) analysis on day 9 post restimulation. (C) control CTL line stained with H-2K<sup>b</sup>/βgal<sub>96-103</sub> tetramer. (upper quadrant shows percentage tetramer + / CD8 + cells )

### 3.4 $\beta$ gal specific CTL recognise $\beta$ gal expressing Z16 tumour cells in vitro

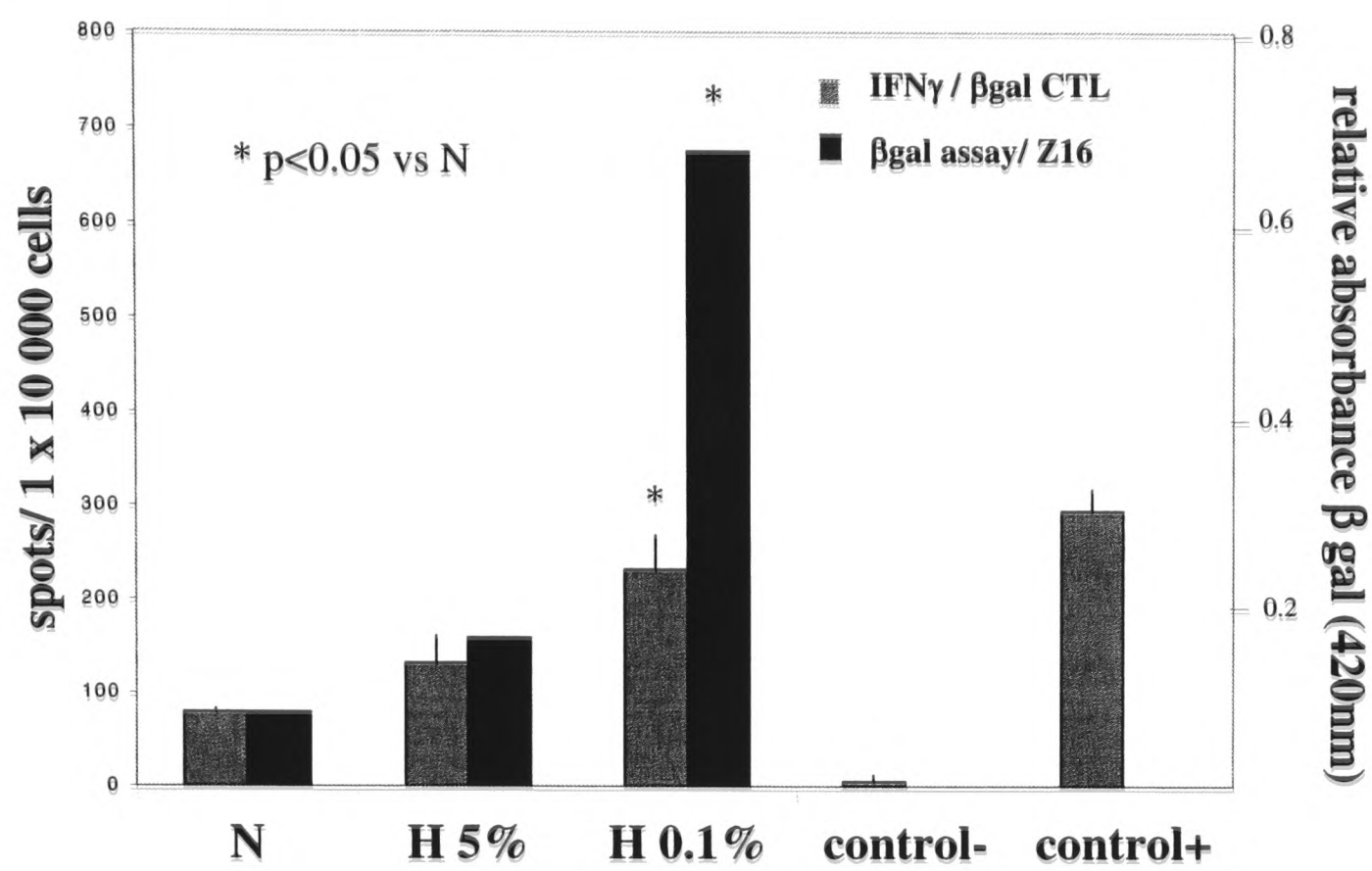
To determine whether  $\beta$ gal expressed by Z16 tumour cells could activate  $\beta$ gal specific CTL in vitro, these CTL were co-incubated overnight with unirradiated Z16 tumour cells that were previously cultured in normoxia or hypoxia (16 hours). Activation of  $\beta$ gal specific CTL was determined by way of IFN $\gamma$  secretion, and analysed in a IFN $\gamma$  ELIspot assay.

Fig 3.4.1 shows the tetramer staining of a population of  $\beta$ gal specific CTL expanded in vitro from 2  $\beta$ gal vaccinated mice. Subsequent ELIspot analysis showed a significantly greater number of IFN $\gamma$  spots from recognition of  $\beta$ gal antigen in hypoxic vs normoxic Z16 by  $\beta$ gal specific CTL effectors ( $p < 0.05$ ). There was no difference in IFN $\gamma$  expression from CTL incubated with wild type B16 tumour cells under similar hypoxic and normoxic conditions. CTL effectors alone or pulsed with the cognate peptide served as negative and positive controls respectively.

In Fig 3.4.2 a relationship is demonstrated between the level of  $\beta$ gal expression in Z16 tumour cells, and recognition of the cognate peptide endogenously presented on the same tumour cells by  $\beta$ gal specific CTL.  $\beta$ gal expression was induced to varying levels in Z16 tumours by pre-incubating in normoxia and hypoxia (5% and 0.1% oxygen tension). This was measured as described previously using  $\beta$ gal substrate read spectrophotometrically at 420nm. The same Z16 targets were co-incubated with  $\beta$ gal specific CTL effectors overnight. IFN $\gamma$  release was quantified by the IFN $\gamma$  ELIspot assay and directly compared with levels of  $\beta$ gal induction. There was a significant expression of IFN $\gamma$  by  $\beta$ gal specific



**Fig 3.4.1 In vitro restimulated  $\beta gal$  specific CTL recognise  $\beta gal$  expressed by Z16 tumour cells under hypoxic conditions.** Upper FACS plots show  $\beta gal$  specific CTL generated as previously in 2 mice, and analysed on day 9 post in vitro restimulation by tetramer/ CD8 staining and flow cytometry (*Upper quadrant shows percentage tetramer +/ CD8+ cells*). Lower bar charts show IFN $\gamma$  ELISpot data following 16 hours co-incubation of CTL and targets (B16 and Z16) at E:T ratio 1:10 and 1:2 for mouse 1 and 2 respectively. Control wells contain CTL alone (control -) or pulsed with 10 $\mu$ M cognate  $\beta gal$  peptide (control +). N =normoxia, H = hypoxia for 16 hours at 1.0% oxygen tension. (Mean values of duplicate wells with 1 SD shown. \* Comparison of means using 2-tailed t test)



**Fig 3.4.2 Recognition of βgal<sub>96-103</sub> peptide by in vitro restimulated βgal specific CTL correlates with βgal expression by Z16 tumours.** Grey bars show IFNγ ELISpot data from βgal specific CTL from one mouse mixed with Z16 tumours (E:T ratio 1:10) pre-incubated under varying conditions. Control wells contain CTL alone (control-) or pulsed with 10μM cognate βgal peptide (control+). N=normoxia, H=hypoxia with figures showing % oxygen tension. (*Mean values of duplicate wells with 1 SD shown.*  
\**Comparison of means using one way ANOVA and Dunnett's multiple comparisons test*). Corresponding black bars represent relative βgal assay levels from the same tumour cells used for ELISpot analysis. βgal levels were normalised to ELISpot levels in normoxia.

CTL at 0.1% hypoxia over normoxic controls ( $p < 0.05$ ), with a positive correlation between  $\beta$ gal expression by Z16 and IFN $\gamma$  release by CTL although the relationship was not linear. CTL effectors alone or pulsed with the cognate peptide served as controls.

The data show that Z16 tumours express increased levels of  $\beta$ gal under hypoxia, which translates into increased presentation of peptide/MHC I and recognition by  $\beta$ gal specific CTL.

### **3.5 Vaccination against hypoxia-regulated tumour antigen $\beta$ gal partially protects mice from Z16 subcutaneous tumour challenge**

Following the successful induction of a  $\beta$ gal specific CTL response *in vitro*, it was hypothesised that such a response *in vivo* could afford protection against a tumour expressing  $\beta$ gal under hypoxic conditions.

$\beta$ gal specific CTL responses were induced in C57BL/6 mice through intravenous priming with  $\beta$ gal protein and  $\alpha$  GalCer. Boosting was performed 7 days later with intravenous recombinant vaccinia virus encoding the full length  $\beta$ gal gene. Six days later the mice were challenged with Z16 tumour cells injected into the left flanks. As controls, the right flanks were simultaneously injected with wild type B16 tumour cells. Measurements of tumour growth were taken as described in Materials and Methods, twice a week from the second week onwards.



The mice immunised against  $\beta$ gal showed significantly delayed Z16 tumour engraftment compared to naïve mice at day 14 and 18 post challenge ( $p < 0.05$ ). However, tumour growth continued exponentially for both groups from the third week. Mean Z16 tumour growth in naïve mice was not different to that of wild type B16 tumours (fig 3.5). Therefore vaccination for  $\beta$ gal tumour antigen only partially protected mice challenged with hypoxia regulated  $\beta$ gal expressing Z16 tumours.

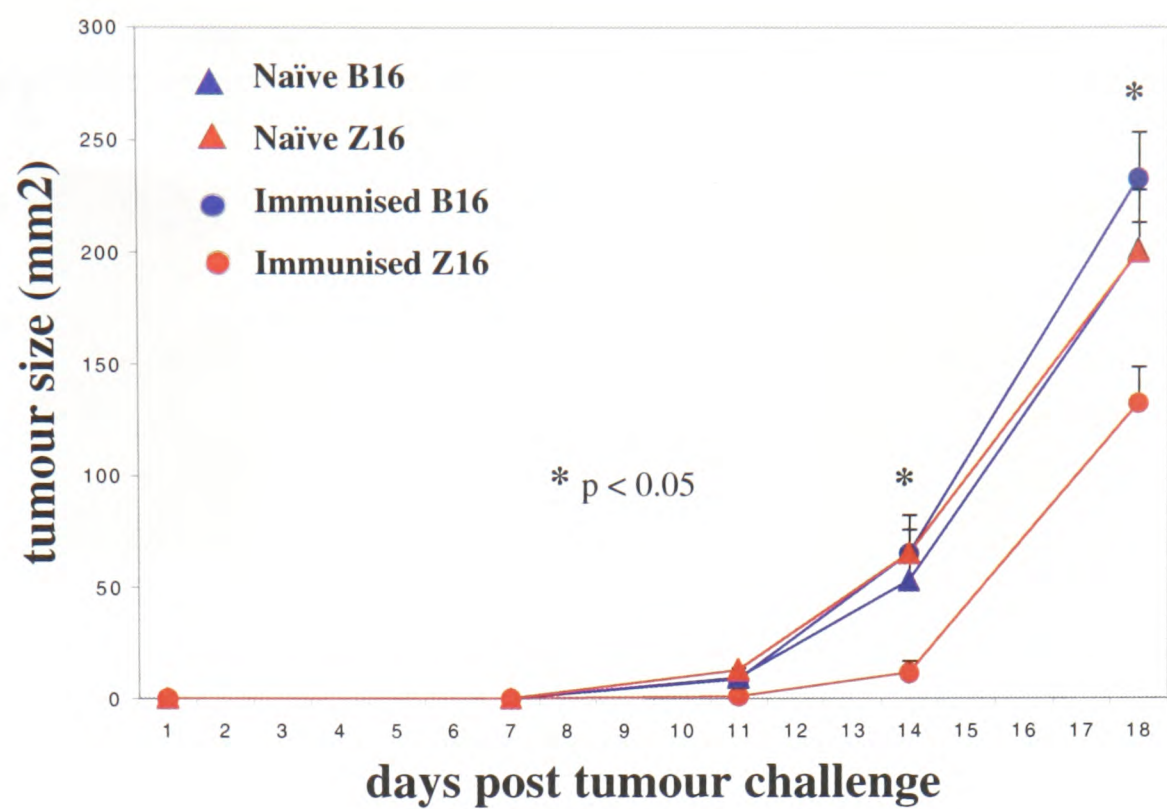
### **3.6 Z16 subcutaneous tumour challenge boosts $\beta$ gal specific CTL responses in immunised mice**

Partial protection against Z16 tumours by  $\beta$ gal specific CTL implied recognition of target cells by effector T lymphocytes *in vivo*. Further evidence that  $\beta$ gal specific CTL interacted with Z16 tumours was provided by the boosting of  $\beta$ gal specific CTL responses detected *ex vivo*. Immunised mice bearing subcutaneous Z16 tumours were tail-bled at day -1 pre and day 11 post tumour challenge, with the  $\beta$ gal specific CTL population analysed by tetramer staining and flow cytometry. All immunised mice bearing Z16 tumours showed increase in  $\beta$ gal specific CTL populations (fig3.6), while unimmunised mice similarly challenged did not show any increases (data not shown).

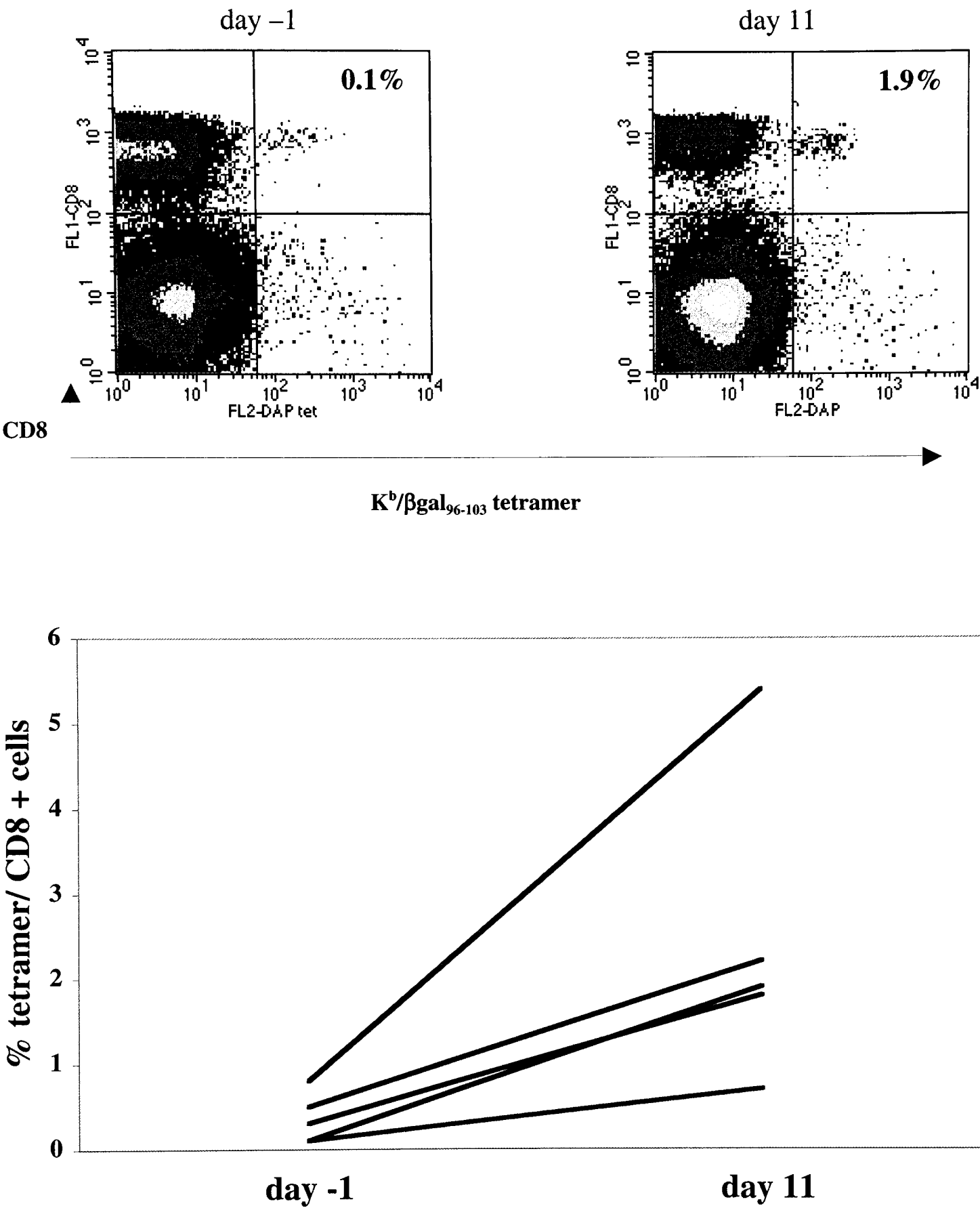
### **3.7 Vaccination against hypoxia-regulated tumour antigen $\beta$ gal protects mice from Z16 pulmonary metastases challenge**

To demonstrate that vaccination against  $\beta$ gal could protect mice from Z16 pulmonary metastases challenge, mice were immunised against  $\beta$ gal as above, and subjected to intravenous Z16 tumour challenge on day 8 following the last immunisation. Naïve mice





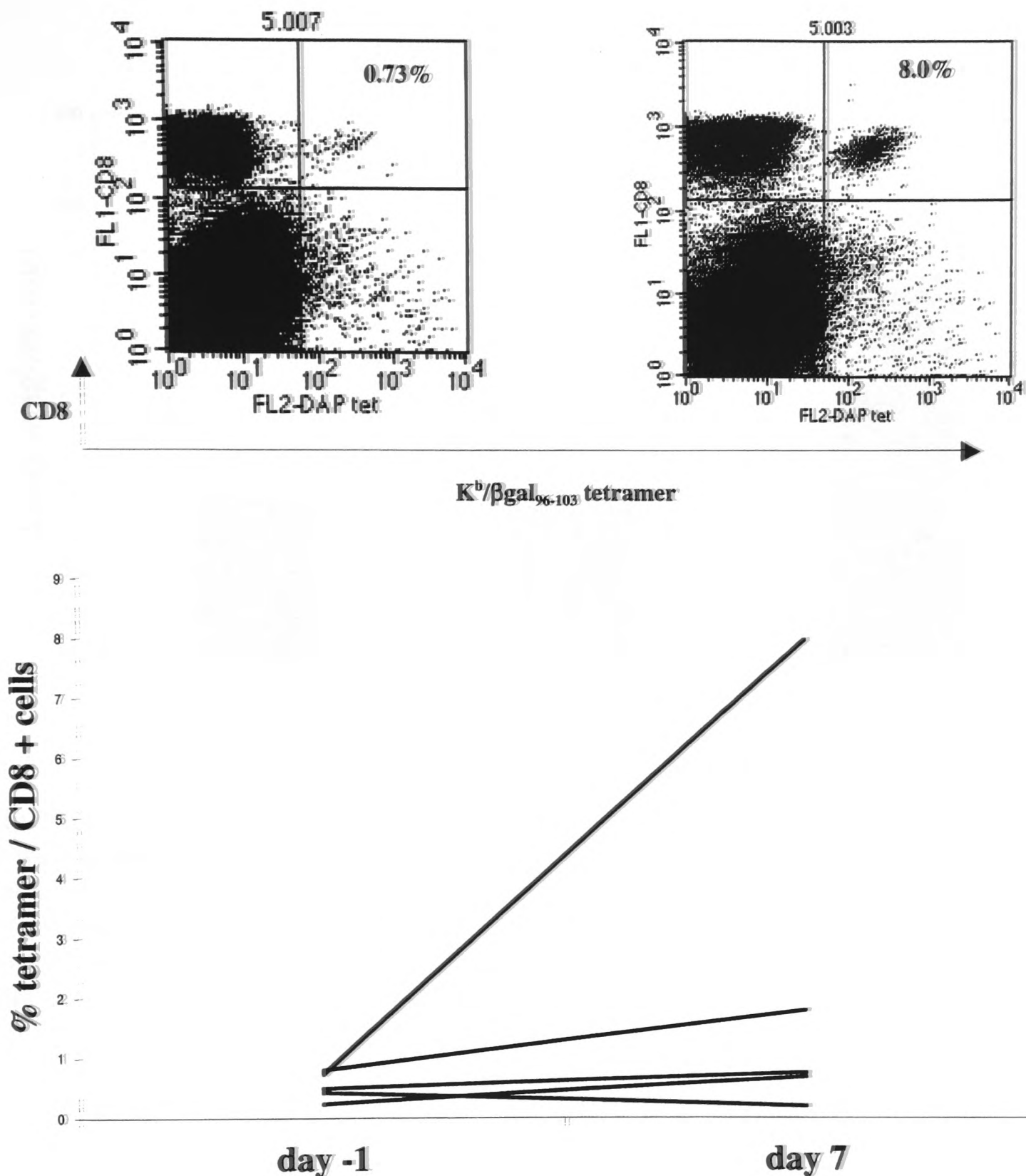
**Fig 3.5 Vaccination against hypoxia-regulated tumour antigen  $\beta$ gal partially protects mice from Z16 subcutaneous tumour challenge** Growth curves show B16 and Z16 tumour progression in naïve and immunised mice injected subcutaneously with tumour. Measurements were taken twice a week, and tumour size for each group was calculated as the mean of the products of bisecting diameters (+/- SD). Measurements were terminated for each group when the first animal developed a tumour in excess of 200 mm<sup>2</sup>. Blue curves = B16 tumours, red curves = Z16 tumours; triangle = naïve, dot = immunised. ( Mean values with 1 SD shown, 5 mice per group. \*Comparison of means using one way ANOVA)



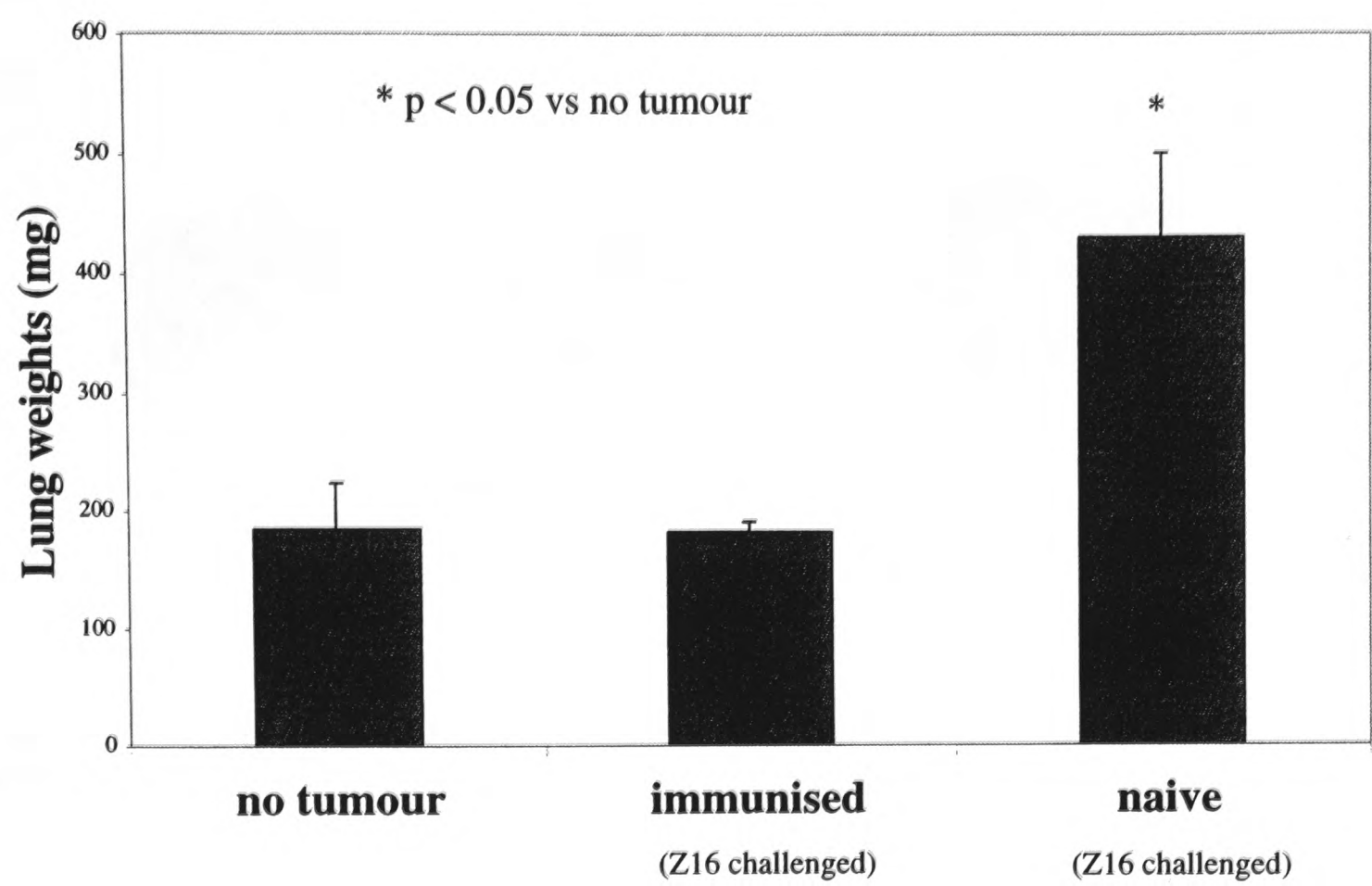
**Fig 3.6 Z16 subcutaneous tumour challenge boosts βgal specific CTL in vivo** Upper FACS plots representative of *ex vivo* tetramer analysis of βgal specific CTL from peripheral blood lymphocytes on day-1 pre- (left) and day 11 post-tumour (right) challenge. (*right upper quadrant show percentage tetramer/ CD8 + cells*). Lower plot shows increase in percentage tetramer/ CD8 + cells from day -1 to day 11 post tumour challenge, each line representing a mouse

were similarly challenged as controls. Mice were tail-bled on day 7 post tumour challenge, and PBL stained with H-2K<sup>b</sup>/βgal<sub>96-103</sub> tetramer and analysed by flow cytometry. On day 24 post tumour challenge, all mice were sacrificed and lungs harvested. Lung weights were compared with those from naïve unchallenged mice.

Tetramer analysis of PBL on day 7 post tumour challenge showed increase in the tetramer + population except in 1 mouse (fig 3.7.1). Lung weights in immunised mice were similar to those of unchallenged mice 24 days after tumour challenge, with lungs from naïve mice significantly increased ( $p < 0.05$ ) over lungs from unchallenged mice (fig 3.7.2). However, this protection in immunised mice was not complete, since there were areas of metastases evident on lung specimens *ex vivo* (fig 3.7.3). Therefore vaccination for βgal tumour antigen again only partially protected mice challenged with Z16 pulmonary metastases.



**Fig 3.7.1 Z16 intravenous tumour challenge boosts  $\beta$ gal specific CTL in vivo** Upper FACS plots representative of *ex vivo* tetramer analysis of  $\beta$ gal specific CTL from peripheral blood lymphocytes from a tail-bled immunised mouse on day -1 before (left) and day 7 after (right) tumour challenge. (right upper quadrant show percentage tetramer/ CD8 + cells). Lower plot shows increase in percentage tetramer/ CD8 + cells from day -1 pre- to day 7 post-tumour challenge, each line representing one mouse.



**Fig 3.7.2 Vaccination against hypoxia-regulated tumour antigen  $\beta$ gal protects mice from Z16 intravenous tumour challenge** Bar charts showing mean lung weights in naïve and immunised mice harvested 24 days following challenge with Z16 tumour intravenously. Comparison made with lung weights from naïve, unchallenged (no tumour) mice. (Mean values with 1 SD shown, 5 mice per group. \*Comparison of means using one way ANOVA with Dunnetts multiple comparisons test)



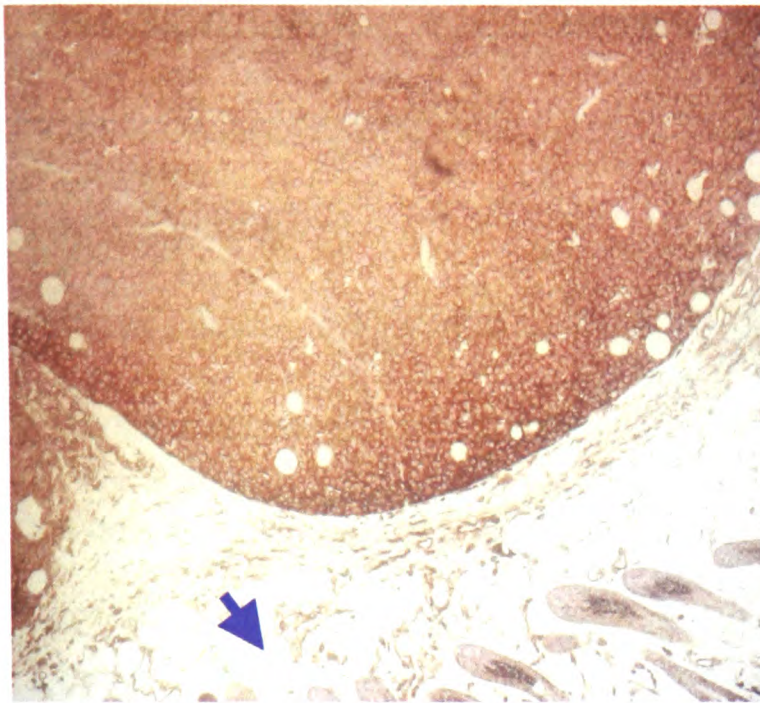
**Fig 3.7.3 Vaccination against hypoxia-regulated tumour antigen  $\beta$ gal protects mice from intravenous Z16 tumour challenge** Representative lung specimens harvested from Z16 tumour challenge in immunised (centre) and naïve (right) mice. Control mice without tumour challenge (left).

### **3.8 Immunohistochemistry shows $\beta$ gal staining in Z16 tumours harvested from C57BL/6 mice**

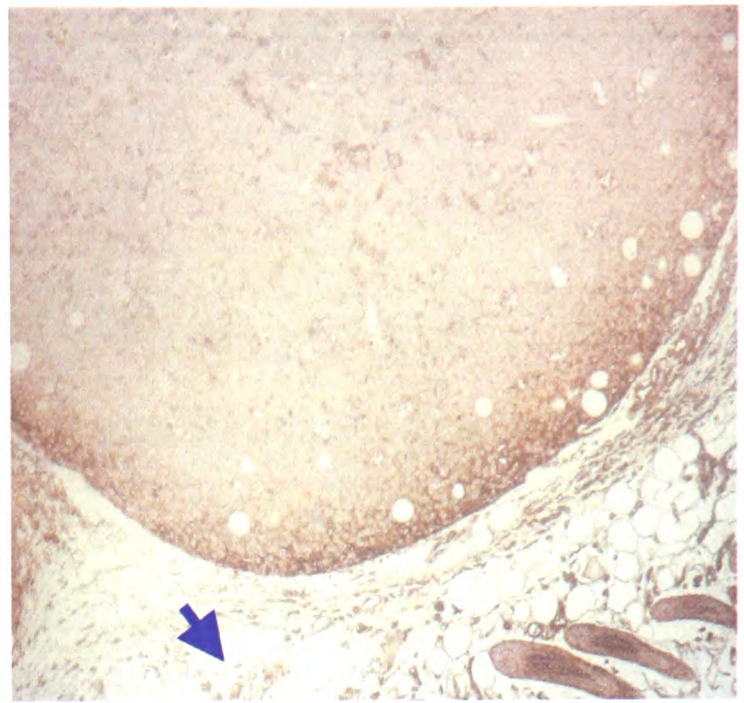
To show that  $\beta$ gal expression was present in Z16 tumours, subcutaneously implanted Z16 and B16/F10 tumours as described in section 3.6 were harvested on day 20 after tumour implantation. The encapsulated tumours appeared to be vascularised from the periphery with a central necrotic core. Tumours were formalin fixed, paraffin embedded, sections cut and mounted. Slides were stained with anti- $\beta$ gal antibody and further developed with as described in Materials and methods. Figure 3.8 A shows expression of  $\beta$ gal (brown stain) throughout the tumour with no staining in the control section (fig 3.8 B). In the immunised mice, there was loss of  $\beta$ gal expressing tumour cells (fig 3.8 C), indicating negative selection against those cells by  $\beta$ gal specific CTL *in vivo*. In comparison, there was no expression of  $\beta$ gal in wild type B16.F10 tumours (fig 3.8 D)



A : naïve/ Z16



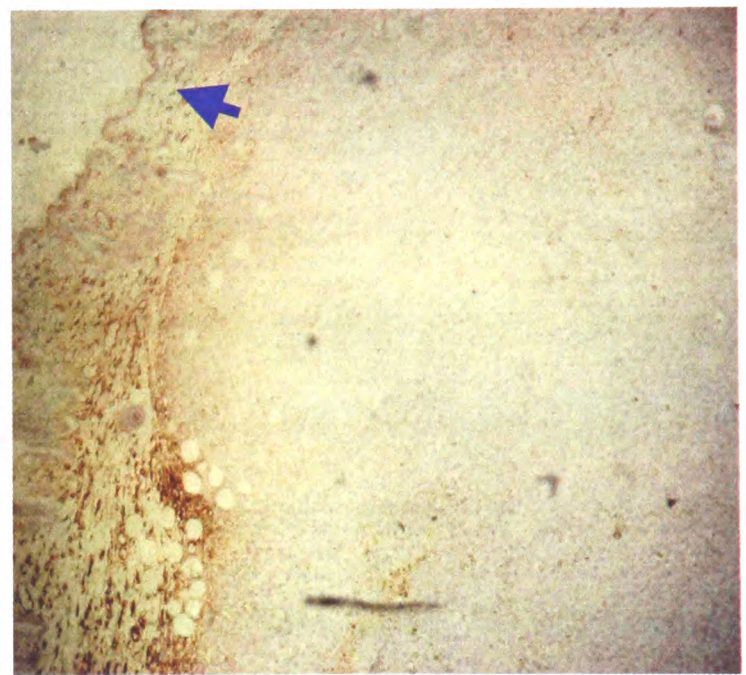
B : naïve/ Z16 (no primary Ab)



C : immunised/ Z16



D : immunised/ B16.F10



**Fig 3.8 Z16 tumour cells express  $\beta$ gal protein *in vivo*.** Z16 and B16.F10 tumours were implanted subcutaneously in C57BL/6 mice. Twenty days later the animals were culled and tumours harvested in formalin and paraffin embedded, with sections cut and mounted. Staining for  $\beta$ gal protein was performed using two-step horseradish peroxidase (HRP) system (brown), and counterstained with hematoxylin. A : Z16 tumours in naïve mice, B : Z16 tumours in naïve mice (control without primary antibody), C : Z16 tumours in  $\beta$ gal immunised mice, D : B16.F10 in immunised mice. ( all sections at 10x magnification; blue arrows indicate skin surface)



### 3.9 Discussion

In these experiments, a  $\beta$ gal expressing murine tumour cell line Z16 was used whose level of  $\beta$ gal induction correlated positively with time (fig 3.2.2) and degree (fig 3.2.1) of hypoxia. The expression of  $\beta$ gal after hypoxic induction was also shown to be stable over time (fig 3.2.3).

Hypoxia-regulated tumour antigen  $\beta$ gal was demonstrated to induce specific T cell responses *in vitro* and *in vivo*, and that these responses resulted in anti-tumour effects. The *in vitro* data demonstrates the induction of specific T cell populations to the cognate peptide  $\beta$ gal<sub>96-103</sub> (fig 3.3), and that IFN $\gamma$  release could be elicited by recognition of hypoxia-driven  $\beta$ gal expression in Z16 tumours (fig 3.4.1). The degree of  $\beta$ gal expression by Z16 tumours correlated positively with IFN $\gamma$  release by  $\beta$ gal specific CTL (fig 3.4.2).

The *ex vivo* data on PBL derived from tail-bleeds in subcutaneous Z16 challenged mice (fig 3.6) suggest that activated  $\beta$ gal specific T cells were restimulated *in vivo*. Harris *et al* (Harris et al., 2002) have reported differential T cell function and fate in lymph node versus non-lymphoid tissue. Activated T cells present in the lymph nodes could be stimulated by antigen to divide, whilst peripheral tissue associated T cells could not. Therefore the boosting of  $\beta$ gal specific CD8 + T cell responses in these experiments implies that the cognate peptide was encountered in the draining lymph node and not in the tumour. The *ex vivo* data was obtained at day 11 post-tumour challenge, at a time when tumours were just palpable. It was not established whether the cognate peptide was presented on Z16 tumour cells that had metastasized, or whether it was cross presented

by professional APC that had taken up  $\beta$ gal protein released from apoptotic Z16 tumour cells in the periphery and migrated to the draining lymph node.

It has been shown that tumour growth beyond a few millimetres already results in diffusion-related hypoxia (Vaupel et al., 2001) as a consequence of the diffusion limits of oxygen (180  $\mu$ m). The tumour challenge data (fig 3.5) implies migration of  $\beta$ gal specific CTL into  $\beta$ gal expressing hypoxic areas of the tumour in order to effect anti-tumour responses. The results of these tumour challenge experiments showed only partial resistance to tumour growth in immunised mice, with a delay of 4-5 days compared with naïve mice. Ultimately, there was tumour escape from the immune response with similar growth rates between groups after the initial delay. The results suggest that the most probable mechanism of tumour escape was the loss of expression of tumour antigen  $\beta$ gal, since Z16 tumour explants stained negative for  $\beta$ gal in immunised mice (fig 3.8). This was likely to have been a result of selective pressures against  $\beta$ gal expressing tumour cells early on during tumour growth, with those cells that had lost expression of the  $\beta$ gal transgene surviving to form the resultant tumour mass. Another possibility would be the different kinetics of tumour growth and destruction by anti-tumour immunity, being faster in the former and eventually overwhelming the immune response. Alternatively, tumour-induced anergy or deletion of effector lymphocytes could have occurred. No functional analyses of those  $\beta$ gal specific T cells following tumour engraftment were performed. Adoptive transfer experiments into immuno-incompetent mice similarly challenged with Z16 tumours could have addressed this issue. That the growth curve of Z16 tumours in naïve hosts was similar to that of wild type B16 in both naïve and

immunised hosts, indicates that the anti-tumour effect was specific to the expression of  $\beta$ gal by the tumour, as well as the presence of  $\beta$ gal specific T cells.

Interestingly in the intravenous pulmonary model, there was more complete protection against tumour development, with lung weights in immunised mice being similar to that of unchallenged mice at day 24 (fig 3.7.2), although they were not absolutely tumour free (fig 3.7.3). There was a similar induction of the  $\beta$ gal specific T cell response (fig 3.7.1), with lower levels of % tetramer +/- CD8 + cells, possibly explained by the earlier time point (day 7) of *ex vivo* analysis compared with the subcutaneous model (day 11). The data suggests that CTL recognition of cognate peptide/MHC I occurred *in vivo*, since it was demonstrated that activation of the CTL memory responses to  $\beta$ gal occurred in immunised mice challenged intravenously with Z16. The superior level of protection seems counter-intuitive since Z16 tumours in the lung would be in a relatively oxygen-rich environment, with decreased induction of  $\beta$ gal tumour antigen. This discrepancy could be explained in part by the different routes of injection and tumour localisation. Intravenously injected lung-implanted tumour cells co-localised with, and were more efficiently exposed to circulating effector CTL resulting in recognition and deletion of  $\beta$ gal expressing tumour cells. The differences in tumour inoculation and localisation could account for different effector T cell trafficking and ultimately tumour rejection. It was not ascertained whether  $\beta$ gal was expressed *in vivo* in the pulmonary Z16 tumours.

Alternatively, the differences in Z16 tumour growths in the lung versus subcutaneously inoculated tumours could be due to differences in the tumour environment per se, such as

the presence of growth factors. Indeed, growth factors and other non-hypoxic pathways have been demonstrated to regulate the expression of HIF-1 $\alpha$  (reviewed by Harris (Harris, 2002), which may have bearing on the induction of  $\beta$ gal expression by Z16 tumour cells.

It was not possible to correlate the levels of  $\beta$ gal induction by Z16 tumours *in vivo*, with *ex vivo* tetramer specific responses or with  $\beta$ gal specific anti-tumour immunity. There was a detectable basal level of  $\beta$ gal expression by Z16 under normoxic conditions (20% oxygen tension) *in vitro*, indicating transcriptional leakage even under restrictive conditions (fig 3.2.1). The basal expression of  $\beta$ gal by Z16 tumours in normoxia means that the *in vivo* induction of  $\beta$ gal specific T cell responses and anti-tumour effects could not be attributed entirely to the effects of hypoxic induction. Ideally, comparisons should be made with different tumours constitutively expressing low and high levels of  $\beta$ gal, and results compared to hypoxia-driven  $\beta$ gal expression in Z16. Nevertheless, the results raise important issues related to hypoxia-regulated tumour antigens. There is the question of the effects of hypoxia on antigen processing and presentation in tumour cells and other APC, and whether these mechanisms could contribute to tumour escape. In particular, the dual pathways of direct and cross-presentation of tumour antigens could play different roles in normoxia and hypoxia. The effect of intra-tumour hypoxia and its inherent vascular instability on T cell migration also needs to be further explored, this being perhaps another means of immune resistance. It is compelling to speculate whether induction of immune responses to hypoxia-driven antigens could extend to self-antigens, and if so, overcome the intrinsic problems of low avidity self-reactive T cells and

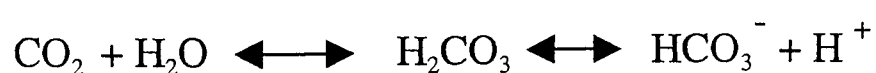
peripheral tolerance mechanisms. Finally, with the development of anti-angiogenic therapies aimed at the tumour vasculature and inducing hypoxic/ anoxic tumour destruction, it would be interesting to look for any synergy between this and immunotherapy directed against hypoxia-driven tumour antigens *in vivo*.

## Chapter 4. Human CA9/G250 specific T cell responses in a transgenic mouse model

### 4.1 Introduction

In this chapter, T cell responses to a human hypoxia-regulated antigen carbonic anhydrase isoenzyme 9 (CA9 or G250) in a transgenic mouse model (HHD) were characterized. HHD mice are murine MHC class I and  $\beta$ 2-microglobulin ( $\beta$ 2-m) double knockouts, and transgenic for the chimeric heavy chain comprised of HLA-A2.1  $\alpha$ 1 and  $\alpha$ 2, H-2D<sup>b</sup>  $\alpha$ 3, transmembrane and cytoplasmic domains, covalently linked to human  $\beta$ 2-m by the amino terminus of the  $\alpha$ 1 domain. In these mice, the transgenic molecules are the only class I molecules detectable at the cell surface (as opposed to A2K<sup>b</sup> transgenics which express endogenous MHC1 in addition to A2K<sup>b</sup>). The presentation of peptides restricted by HLA-A2.1, together with murine CD8 co-receptor binding to H-2D<sup>b</sup>  $\alpha$ 3 domains, allows for efficient *in vivo* induction of CTL responses to HLA-A2.1 restricted peptides and their subsequent analysis.

Carbonic anhydrases (CA) are a family of isoforms of zinc metalloenzymes that catalyse the conversion between carbon dioxide and bicarbonate:



The last 4 years has seen much effort being put into identifying the role of the CA in tumour progression. CA9 has been shown to be strongly induced in tumour hypoxia (Wykoff et al., 2000) and also involved in cell-cell adhesion together with E-cadherin

(Svastova et al., 2003), a point of relevance to hypoxia-induced tumour metastases. Under physiological conditions, CA9 expression was detected in the basal cells of hair follicles, mesothelial linings, epithelial linings of the gallbladder, bile and pancreatic ducts, gastric mucosa, Brunner's glands and crypt cells of the duodenum and jejunum (Ivanov et al., 2001). The regulation of CA9 by hypoxia has been attributed to the transcriptional effects of hypoxia-inducible factor (HIF)-1 $\alpha$  (Wykoff et al., 2000). Kaluz *et al* (Kaluz et al., 2002; Kaluz et al., 2003) have also described an alternative pathway of CA9 induction that is HIF-1 $\alpha$  dependent, but driven by specificity protein (SP) 1, by way of phosphatidylinositol 3'-kinase (PI3K) activation. Rafajova *et al* (Rafajova et al., 2004) noted that intratumoral distribution of CA9 only partially overlapped with other hypoxia regulated proteins vascular endothelial growth factor (VEGF) and glucose transporter (GLUT-1), which might be explained by different half-lives of the proteins. Airley (Airley et al., 2003) however, noted a correlation between GLUT-1 and CA9 expressions in carcinomas of the cervix, suggesting a shared influence under HIF-1.

The expression of CA9 in the specialized cells of the gastrointestinal tract involved in acid/base homeostasis supports the proposed role for CA9 in the maintenance of extracellular acidity in the hypoxic tumour microenvironment (Ivanov et al., 2001). Indeed, the correlation of CA9 expression in a wide range of tumours, with biological behaviour is well documented and expansive (reviewed by Potter (Potter and Harris, 2003) . Particularly interesting, is the association of CA9 expression with the clear cell variant of renal cell cancer (RCC) (Turner et al., 2002; Wiesener et al., 2001). In this, the most common variant of RCC (approximately 90% of all cases), the mutations associated



with the von Hippel-Lindau (VHL) gene lead to abrogation of the proteolytic function of pVHL, stabilization of HIF-1 $\alpha$ , and constitutive expression of CA9 independent of hypoxia.

The reports on targeting CA9 expression in tumours from *in vitro* and translational studies are diverse. Monoclonal antibody mG250 specific for CA9 has been used to detect micrometastases in renal cancer patients (Li et al., 2001a), and  $^{131}\text{I}$  tagged mG250 is currently used in Phase 1 and 2 trials for directed radiotherapy (Steffens et al., 1997). CA9 specific antibody dependent cellular cytotoxicity (ADCC) in lymphokine activated killing (LAK) has been used effectively *in vitro* to target renal cancer cells expressing CA9 (Liu et al., 2002).

Visser et al (Visser et al., 1999) have demonstrated *in vivo* generation of HLA-A2 restricted CA9 specific CTL responses by vaccinating A2K<sup>b</sup> transgenic mice with defined HLA-A2 binding human CA 9 peptides, and using splenocytes from such mice as bulk effectors against specific peptide pulsed targets. Using *in silico* predictive algorithms for HLA-A2 binding motifs coupled with *in vitro* binding data, thirteen CA9 derived peptides of intermediate to high binding affinities were identified. Of these, only four HLA-A2 restricted CA9 peptides were found to be immunogenic in mice viz. CA9<sub>26-35</sub>, CA9<sub>254-262</sub>, CA9<sub>271-279</sub> and CA9<sub>421-429</sub>. To demonstrate endogenous antigen processing and presentation, target cells transfected with cDNA encoding the whole CA9 gene were incubated with splenocytes from vaccinated mice. Out of four possible immunogenic



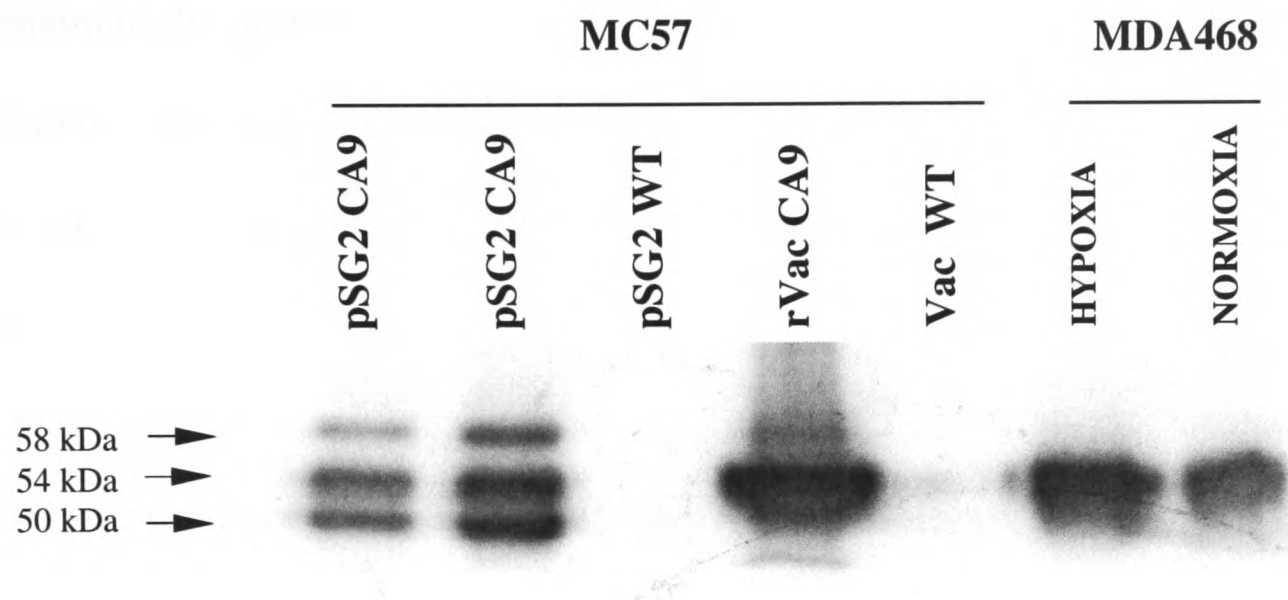
peptides, it was concluded that only the peptide CA9<sub>254-262</sub> was both immunogenic and endogenously processed and presented.

The aims of this chapter include the *in vivo* induction of a high avidity murine T cell line that could specifically recognise the HLA-A2/CA9<sub>254-262</sub> epitope *in vitro*, and to utilise this line to investigate interactions with HLA-A2 restricted human CA9 expressed on target cells. In order to study specific T cell populations, tetramers based on the HLA-A2 restricted CA9<sub>254-262</sub> peptide were generated. To detect specific CTL responses in both human and transgenic HHD mouse systems (see Materials and methods), the tetramers were generated on both HLA-A2 and HLA-A2K<sup>b</sup> MHC I backgrounds respectively. The specificity of the tetramers was ascertained by staining PBL derived from transgenic HHD mice vaccinated for human CA9. The induction of HLA-A2/CA9 responses in transgenic HHD mice was achieved through various vaccination strategies which included the use of recombinant plasmid pSG2 and vaccinia virus encoding for the full length human CA9 product. Using splenocytes harvested from responding mice, a HLA-A2/CA9<sub>254-262</sub> specific T cell line was obtained by *in vitro* restimulation with CA9<sub>254-262</sub> peptide. This line was used to investigate whether specific interactions with endogenously expressed human CA9 could be observed.

## **4.2 Plasmid (pSG2/CA9) transfected and recombinant vaccinia (rVac/CA9) infected murine fibroblasts MC57 express human CA9.**

It has previously been shown that an immunization strategy involving sequential injection of recombinant DNA and vaccinia vectors encoding a specific epitope can elicit powerful CTL-mediated immune responses to various pathogens (Hanke et al., 1999; Kent et al., 1998; Schneider et al., 1998). This potent ‘prime-boost’ immunisation strategy was employed together with other immunisation protocols to induce specific T cell responses *in vivo* in HHD transgenic mice (see section 4.3). Recombinant technology was used to generate a plasmid sequence encoding for human CA9 for DNA immunisation (priming), together with a vaccinia virus encoding for the same product as an immunological ‘boosting’ reagent. To assess the integrity of the recombinant plasmid pSG2/CA9 and recombinant vaccinia encoding for human CA9 (rVac/CA9), a murine fibroblast line MC57 was transiently transfected with pSG2/CA9, and also infected with rVac/CA9 as described in Materials and methods. MC57 cells were chosen for their negative background expression of human CA9 protein. Cell lysates were normalized to 1mg/ml total protein concentration and equal volumes loaded onto a reducing polyacrilamide gel. Blots were stained with M75 antibody specific for the extracellular proteoglycan region of CA9 (Zavada et al., 2000). These were compared with MC57 cells transfected with either wild type pSG2 or infected with wild type vaccinia. As a control for the M75 antibody, hypoxia-induced CA9 from the MDA468 breast tumour line was used (fig 4.2).

The blots in Fig 4.2 showed induction of CA9 by pSG2/CA9 transfected, and rVac/CA9 infected MC57 compared with wild type controls. MDA468 breast tumour lines showed



**Fig 4.2 Plasmid (pSG2 CA9) transfected and vaccinia (rVac CA9) infected murine fibroblasts MC57 express human CA9.** Western blot showing lysates from MC57 cells transfected with pSG2 CA9 (in duplicate) and infected with recombinant vaccinia encoding full length human CA9 (rVac/CA9). Blots were stained with M75 antibody specific for human CA9. Comparisons were made with wild type plasmid (pSG2 WT) and vaccinia (Vac WT). Human breast tumour line MDA468 expressing CA9 under hypoxia (0.1% oxygen tension for 16 hours) used as control. All lysates were adjusted to 1mg total protein per ml and loaded in equal volumes.

induction of CA9 in hypoxia. CA9 appears on Western blots as a protein doublet (54 and 58 kDa), as reported by Pastorekova (Pastorekova et al., 1992) and Zavada (Zavada et al., 1993). A 50kDa form of CA9 has been reported as a soluble cleaved form of CA9 containing the proteoglycan domain, found in the culture media (Zavada et al., 2003). Therefore, both recombinant plasmid pSG2/CA9 and virus rVac/CA9 were able to induce human CA9 expression in target cells. The expression of only the 54 kDa isoform in MDA468 cells may be peculiar to the tumour cell.

#### **4.3 Vaccination of transgenic HHD mice against human CA9 induces a CA9 specific CTL response detectable by novel A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer**

Since only the CA9<sub>254-262</sub> peptide has been shown to be endogenously presented, CA9<sub>254-262</sub> specific responses were induced *in vivo* and tetramers used for detection. It has been previously shown that CA9 specific CTL responses can be raised in A2K<sup>b</sup> transgenic mice, and in PBL from HLA-A2 healthy donors (Vissers et al., 1999). Thus, in order to elicit a CA9<sub>254-262</sub> specific CTL response for validation of the specificity of the A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer, CD8<sup>+</sup> T cell responses were induced in transgenic HHD mice by various protocols (Table 4.3). In HHD mice, the  $\alpha 3$  domain is derived from the MHC allele H-2D<sup>b</sup> which shares over 90% sequence homology with the H-2K<sup>b</sup>  $\alpha 3$  domain (Weiss et al., 1983). Therefore the hybrid A2K<sup>b</sup> tetramer (chimeric human-murine tetramers containing  $\alpha 1/\alpha 2$  domains from HLA-A2 molecules and the  $\alpha 3$  domain from H-2K<sup>b</sup>) is able to bind with HHD CD8 co-receptor, and has been used to stain both high and low avidity T cells in HHD mice in a CD8 independent and dependent manner respectively (Choi et al., 2002), as depicted in the following figure:

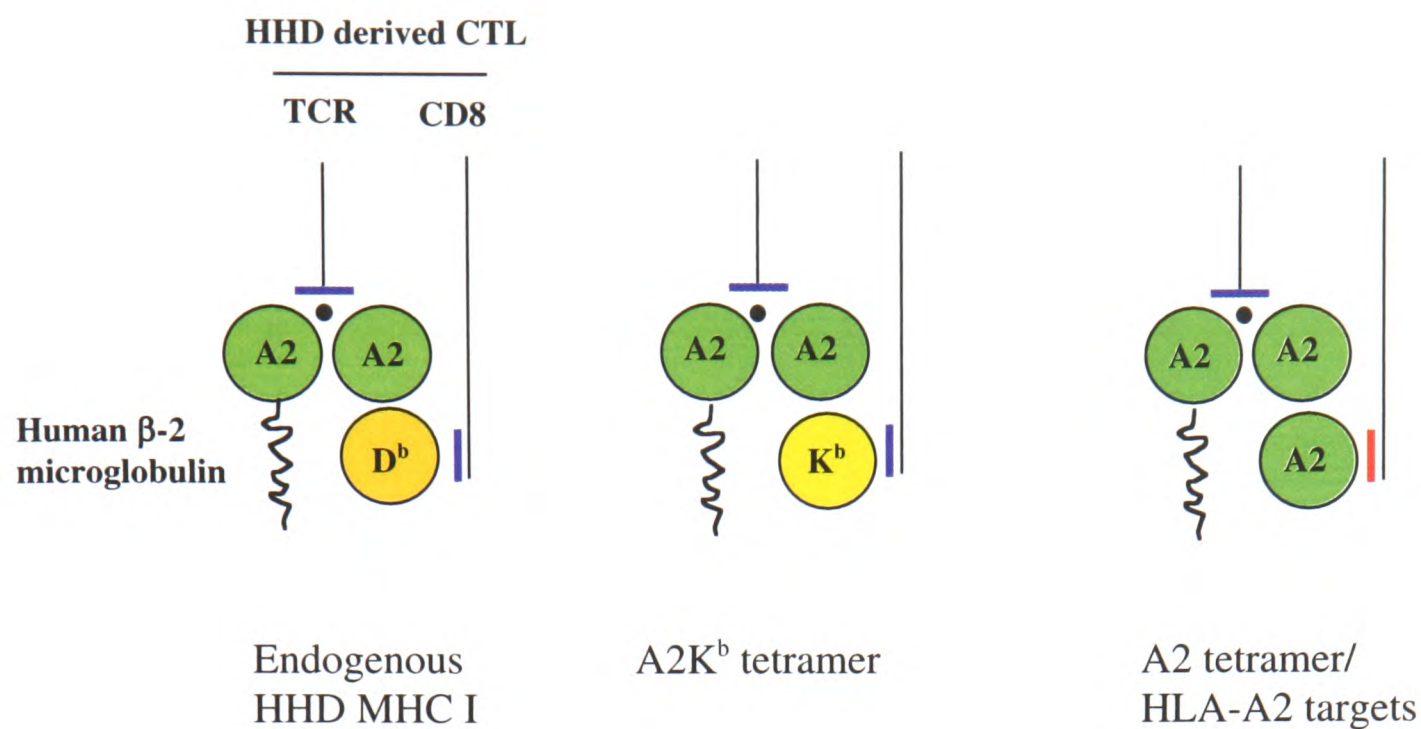


Diagram showing HHD derived CTL TCR binding variously to peptide-MHC complexes from endogenous HHD MHC I, A2K<sup>b</sup> tetramer, A2 tetramer and HLA-A2 targets. MHC I  $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 3 domains in coloured circles depicting HLA-A2 (green), H2-D<sup>b</sup> (orange) and H2-K<sup>b</sup> (yellow); HLA-A2 restricted peptide●, binding interaction —, non-binding interaction —

**Table 4.3 Induction of CD8<sup>+</sup> responses to human CA9 derived peptide (CA9<sub>254-262</sub>) in vaccinated HHD mice**

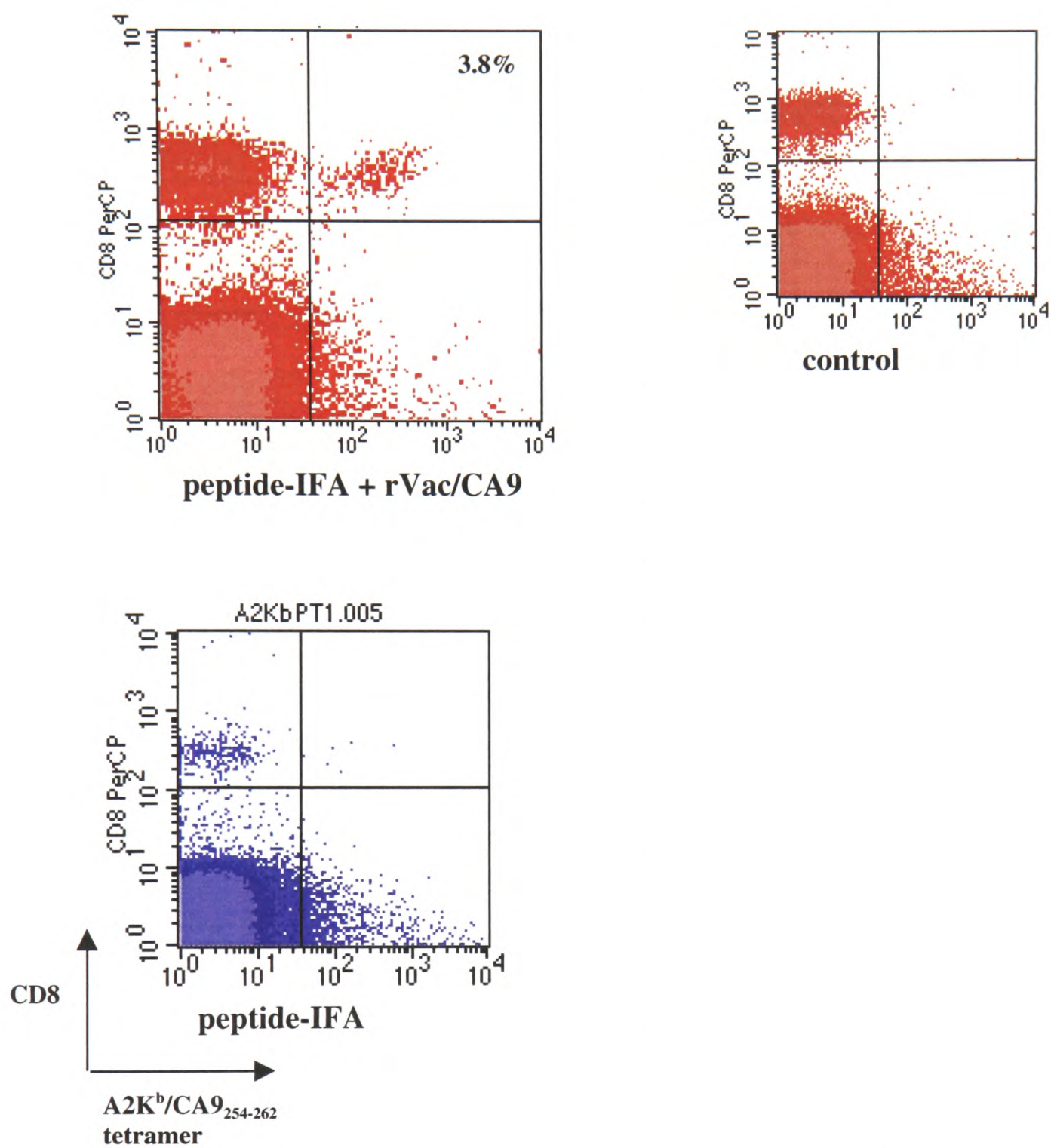
| Cohort<br>(n = no. of mice) | day 0             | day 8        | day 21           | A2K <sup>b</sup> tetramer responses<br>(no. of mice responding/ total) |
|-----------------------------|-------------------|--------------|------------------|------------------------------------------------------------------------|
| A. n = 10                   | i/m DNA(pSG2/CA9) | -            | i/v rVac/CA9     | 0/10                                                                   |
| B. n = 10                   | s/c peptide-BMDC  | i/v rVac/CA9 | -                | 0/10                                                                   |
| C. n = 12                   | s/c peptide-IFA   | i/v rVac/CA9 | -                | 1/12 (fig 4.3.1)                                                       |
| D. n = 13                   | i/m DNA(pSG2/CA9) | -            | s/c peptide-BMDC | 10/13 (fig 4.3.2)                                                      |

Cohort A were subjected to the heterologous prime/boost technique of DNA vaccination involving i/m DNA (pSG2/CA9) injection followed 3 weeks later by i/v rVac/CA9 encoding the same construct. Cohort B were vaccinated s/c with syngeneic bone marrow-derived dendritic cells (BMDC) matured ex vivo and pulsed with CA9<sub>254-262</sub> peptide. These were boosted at day 8 from last vaccination, with rVac/CA9 injected intravenously. Cohort C received subcutaneous CA9<sub>254-262</sub> peptide injection with incomplete Freund’s adjuvant (IFA). This was boosted by i/v rVac/CA9 injection 8 days later. Cohort D received i/m DNA (pSG2/CA9) followed by s/c CA9<sub>254-262</sub> peptide-pulsed BMDC 3 weeks later.

The results in Table 4.3 show that ‘prime-boost’ with recombinant DNA followed by viral immunisation did not elicit any responses detectable by tetramer, suggesting that CA9<sub>254-262</sub> epitope may not have been correctly processed and presented. Priming with peptide-loaded BMDC followed by recombinant viral immunisation similarly failed to induce responses. In one of twelve mice immunised by priming with peptide and adjuvant followed by recombinant viral immunisation, there was a detectable specific response comprising 3.8% of total CD8 cells (fig 4.3.1). This suggests that recombinant CA9 virus immunisation was capable of processing and presentation of the cognate peptide, since peptide and adjuvant alone failed to elicit any responses. Interestingly, recombinant DNA followed by peptide loaded BMDC immunisation evoked detectable responses in the majority of vaccinated mice, and also led to a greater proportion of specific CD8 T cells responses (up to 21.5% in Fig 4.3.2). This also suggests that recombinant DNA immunisation at priming was correctly processed and presented.

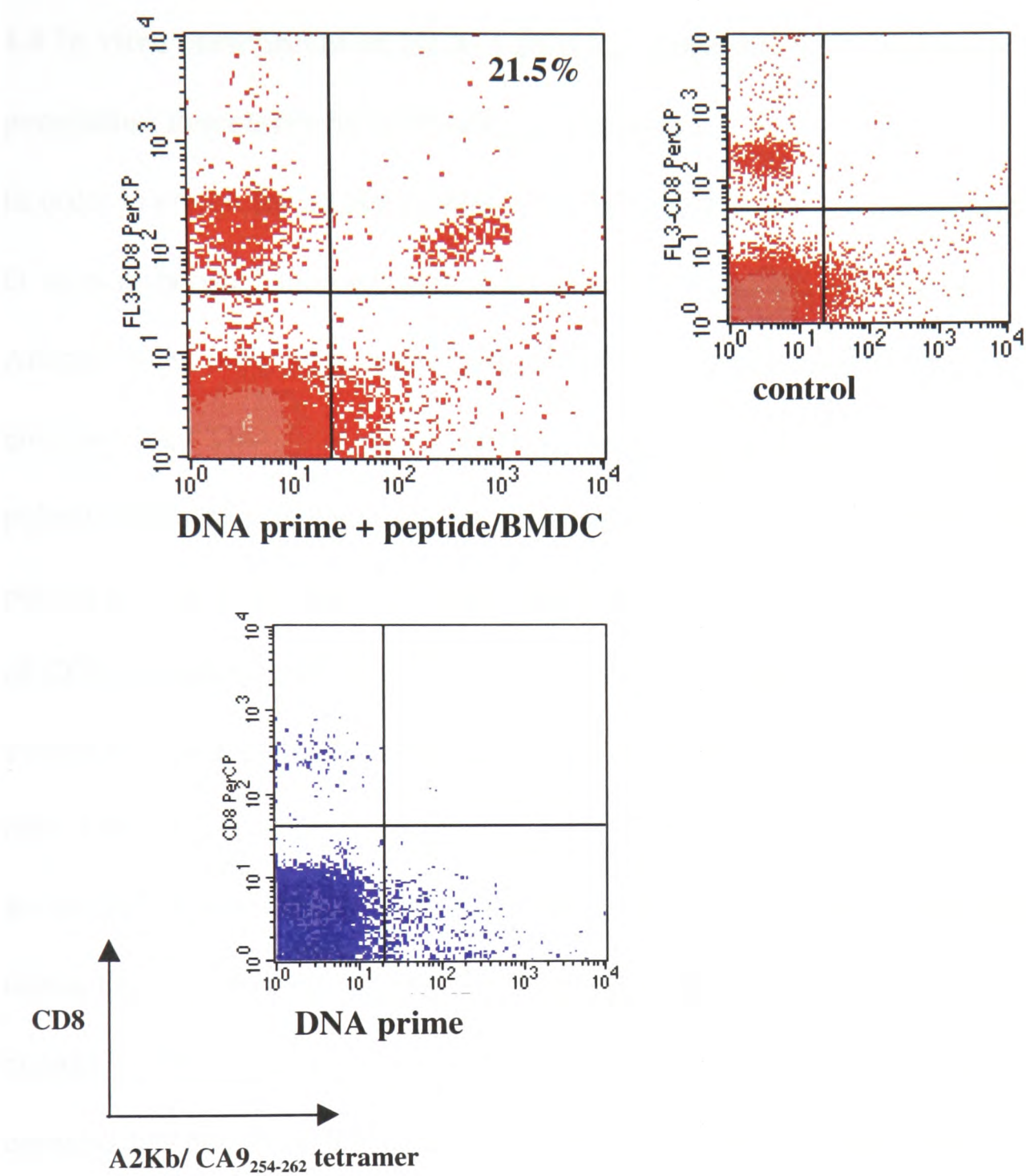
The results in fig 4.3.1 and 4.3.2 validate the specificity of the A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer. No cross-reactivity with control PBL from HHD mice vaccinated for an unrelated antigen Melan-A. Fig 4.3.1 shows the detection of a tetramer +/ CD8 + population (3.8%) by A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer in one of twelve mice in the peptide-IFA prime rVac/CA9 boost group (cohort C). There was no detectable response when vaccinated by peptide-IFA priming alone. In fig 4.3.2, the A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer detected a tetramer +/ CD8 + population (up to 21.5%) in ten of thirteen mice in the DNA prime, peptide-pulsed BMDC boost group (cohort D), with no response with DNA priming alone.





**Fig 4.3.1 CA9<sub>254-262</sub> peptide-IFA prime followed by rVac/CA9 boost induces A2K<sup>b</sup>/CA9<sub>254-262</sub> specific responses in HHD mice.** FACS plots of PBL derived from tail bleeds of one vaccinated mouse, stained with A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer (red plot). Comparisons were made with the same mouse vaccinated earlier with peptide-IFA prime alone (blue plot), and another control mouse vaccinated for an irrelevant antigen (melA) and processed in a similar fashion. (*numbers in upper right quadrant = % tetramer + / CD8 + cells*)



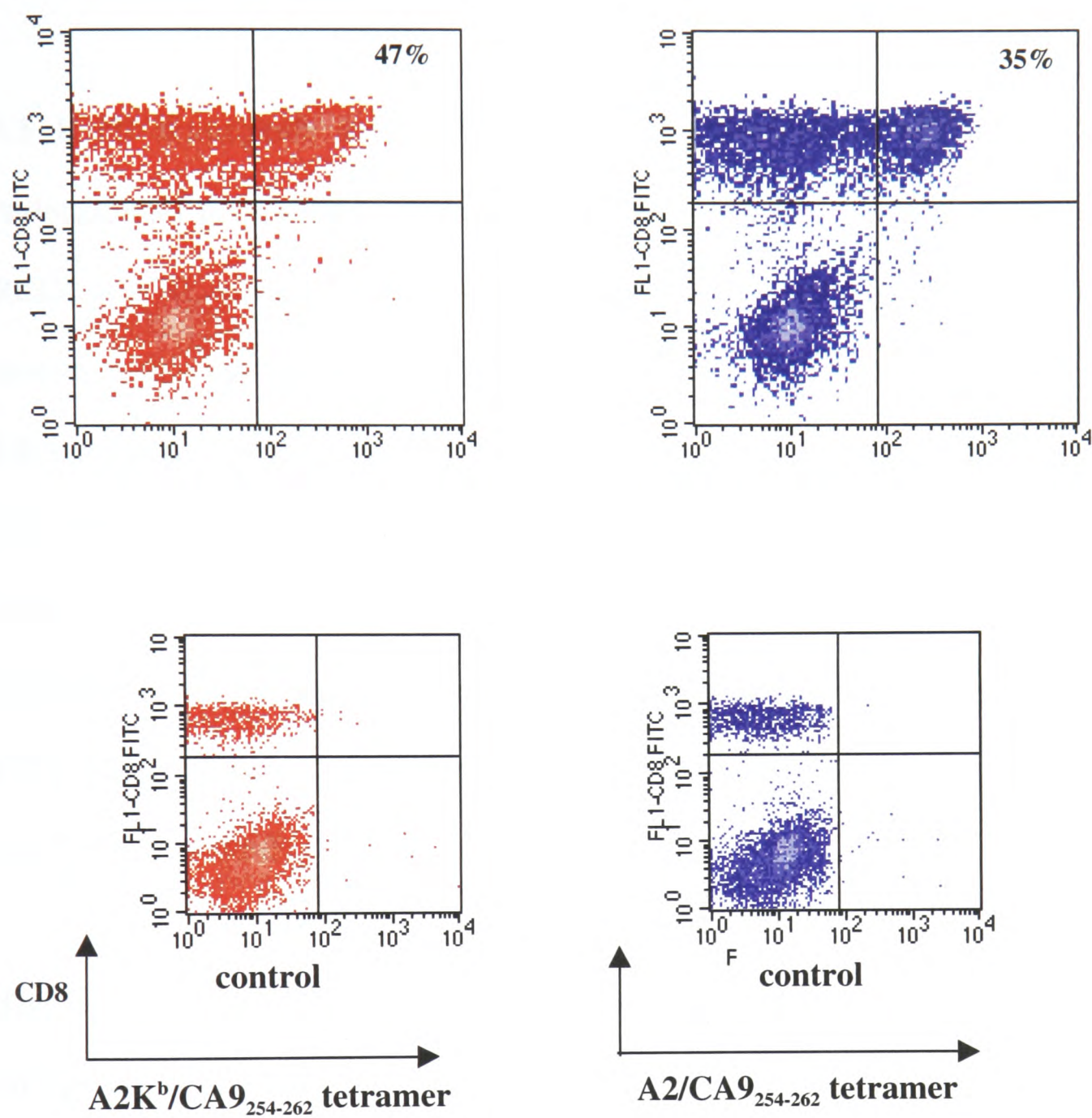


**Fig 4.3.2 CA9 DNA prime followed by CA9<sub>254-262</sub> peptide pulsed BMDC boost induces A2K<sup>b</sup>/CA9<sub>254-262</sub> specific responses in HHD mice.** Representative FACS plots of PBL derived from tail bleed of one vaccinated mouse (cohort D), stained with A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer (red plot) 7 days after last boost. Comparisons were made with same mouse vaccinated earlier with DNA prime alone (blue plot), and control mouse vaccinated for an irrelevant antigen Melan-A and processed in a similar fashion. (numbers in upper right quadrant = % tetramer+ / CD8+ cells)

Therefore, the specificity of the A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer was confirmed through *ex vivo* staining of PBL derived from HHD transgenic mice vaccinated for human CA9.

#### **4.4 In vitro enrichment of HLA-A2/CA9<sub>254-262</sub> specific CTL results in a high avidity population detectable by A2/CA9<sub>254-262</sub> tetramer**

In order to enrich for a CA9 specific CTL line in vitro, the ten responding mice in cohort D were re-boosted intravenously with rVac/CA9 seven days after the last vaccination. Another week later, all animals were sacrificed and spleens harvested. Splenocytes were enriched for CD8<sup>+</sup> /tetramer<sup>+</sup> cells by restimulation in vitro with CA9<sub>254-262</sub> peptide-pulsed irradiated syngeneic splenocytes from a naïve mouse. Tetramer analysis on day 7 post in vitro restimulation using the A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer showed selective expansion of CD8<sup>+</sup>/tetramer<sup>+</sup> cells (fig 4.4 red plot). Choi and others have demonstrated that high avidity CD8<sup>+</sup> + T cells could be identified through selective tetramer staining that did not bind CD8 co-receptor (Choi et al., 2003; Pittet et al., 2003). It was reasoned that successful TCR binding by pMHC tetramer that was CD8 independent implied a stronger interaction and therefore higher avidity. Since a HLA-A2 based tetramer would fail to engage the HHD CD8 co-receptor which recognises both H-2D<sup>b</sup> and K<sup>b</sup> murine  $\alpha$ 3 domains but not HLA-A2 human  $\alpha$ 3 domains, staining of HHD derived CD8<sup>+</sup> + T cells with A2 tetramers could identify higher avidity subpopulations. Fig 4.4 shows the same population of restimulated splenocytes stained with the A2/CA9<sub>254-262</sub> tetramer (fig 4.4 blue plot). The data suggests that of the 47% of CD8<sup>+</sup> + A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer<sup>+</sup> cells, 35% were detectable with CD8 independent tetramer staining, indicating a higher avidity phenotype.



**Fig 4.4 HHD derived CA9<sub>254-262</sub> specific CTL enriched in vitro are stained by A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer and A2/CA9<sub>254-262</sub> tetramer.** Representative FACS plots showing splenocytes derived from one vaccinated mouse (cohort D) restimulated in vitro with CA9<sub>254-262</sub> peptide pulsed irradiated syngeneic splenocytes and stained at day 7 post-restimulation, with A2K<sup>b</sup>/CA9<sub>254-262</sub> tetramer (red plots) or A2/CA9<sub>254-262</sub> tetramer (blue plots). Cells were gated according to controls using HHD mice vaccinated against an irrelevant antigen (UTY). (*numbers in upper right quadrant = % tetramer + / CD8 + cells*)

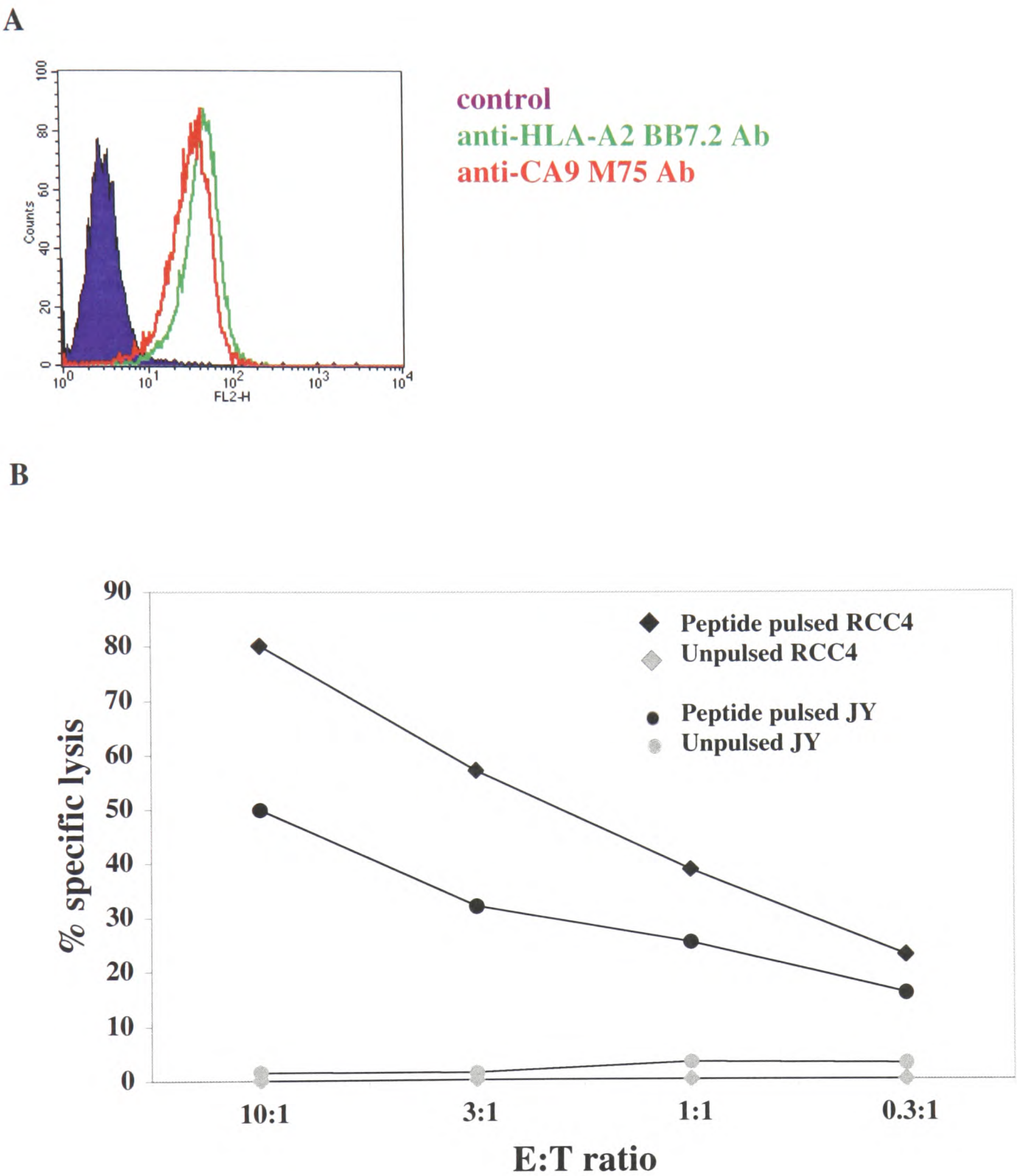
With the identification of high avidity HLA-A2/CA9<sub>254-262</sub> specific T cells derived from HHD mice, it was therefore hypothesised that such effector T cells could recognise endogenously presented human CA9 antigen.

#### **4.5 HHD transgenic mice derived CA9<sub>254-262</sub> specific CTL fail to recognize endogenously presented CA9 peptides on a human tumour cell line RCC4**

RCC4 is a HLA-A2 + human renal cell carcinoma line that is deficient for the VHL protein, with resulting constitutive expression of CA9. This line was confirmed to be both HLA-A2 and CA9 positive by staining with the respective antibodies BB7.2 and M75 (fig 4.5A). To assess for recognition of RCC4 targets by HLA-A2/CA9<sub>254-262</sub> specific T cells derived from transgenic HHD mice, a <sup>51</sup>Cr release cytotoxicity assay was performed. RCC4 cells exogenously loaded with 10 µM CA9<sub>254-262</sub> peptide, and another HLA-A2 + cell line JY (lymphoblastoid human B cell line homozygous for HLA-A2) were used as controls.

The results show that HHD CA9<sub>254-262</sub> specific CTL failed to recognize RCC4 cells endogenously presenting CA9 peptides, but could lyse them if exogenously loaded (Fig 4.5B). Results with peptide pulsed and unpulsed JY targets were similar. The data therefore suggest that despite the identification of high avidity CD8 + T cells with CD8 independent tetramer binding, there was a failure to recognise targets expressing the relevant antigen, which could be attributed to the lack of CD8 binding on target cells or the failure of processing and presentation of cognate peptide by target cells. In either case, high concentrations of exogenously pulsed peptide (10 µM) reversed this.



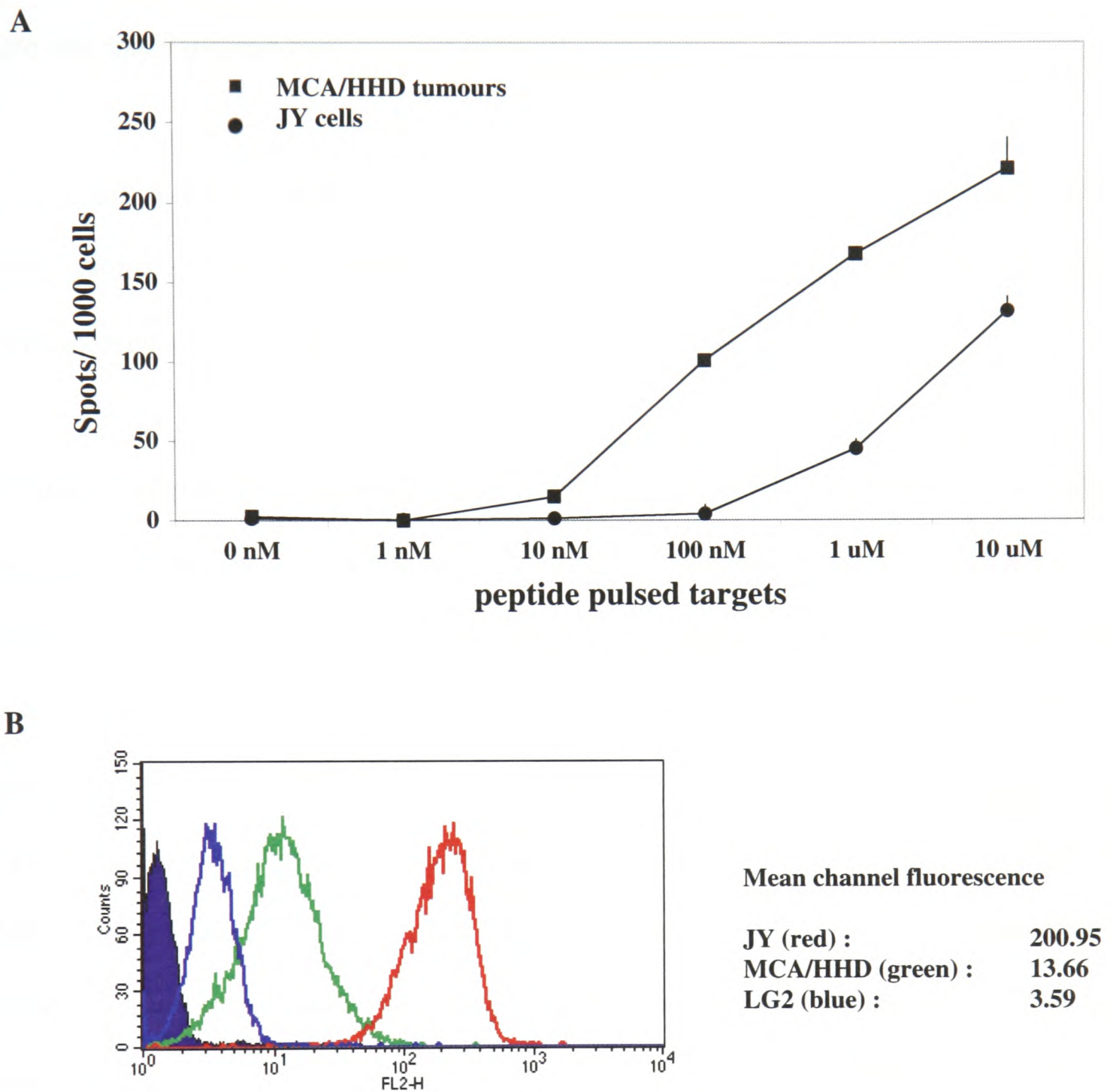


**Fig 4.5 HHD derived CA9<sub>254-262</sub> specific CTL effectors fail to specifically lyse RCC4 human tumour line.** (A) FACS histogram plots of RCC4 stained with specific HLA-A2 antibody BB7.2 (green) and CA9 antibody M75 (red), compared with a HLA-A2-/CA9-negative control (solid purple) (B) <sup>51</sup>Cr release assay to demonstrate specific lysis of RCC4 targets (grey diamond) vs CA9<sub>254-262</sub> peptide pulsed RCC4 (black diamond), and compared with unpulsed (grey circle) and pulsed (black circle) JY targets. All peptides pulsed at 10 μM concentration. (*E:T* = effector:target)

#### 4.6 Avidity of HHD transgenic mice derived HLA-A2/CA9<sub>254-262</sub> specific CTL for CA9<sub>254-262</sub> peptide is dependent on CD8 binding

Although HHD derived CA9<sub>254-262</sub> specific CTL effectors were of high avidity as determined by staining with the A2 tetramer, the failure of these CTL to kill CA9 expressing targets could be a result of the lack of CD8 binding on both RCC4 and JY targets. Since cytotoxicity is one of the outcomes of CTL activation via TCR engagement by peptide/MHC, it was questioned whether the lack of CD8 co-receptor binding made a difference to events consequent to TCR engagement. A head to head comparison of peptide pulsed methylcholanthrene-induced tumours in HHD mice (MCA/HHD) and CD8 non-binding (JY cells) targets looking at TCR downstream events (viz. cytokine release and cytotoxicity) was made.

An IFN $\gamma$  ELISpot assay was used in a CA9<sub>254-262</sub> peptide titration experiment using the same HHD derived HLA-A2/CA9<sub>254-262</sub> specific CTL effectors with peptide pulsed JY or MCA/HHD targets. Fig 4.6.1A shows that a lower amount of peptide pulsed on MCA/HHD cells was required to induce the same level of IFN $\gamma$  ELISpot response as with peptide pulsed JY cells. A possible explanation would be that MCA/HHD targets express higher numbers of peptide/MHC (HLA-A2) complexes. The mean channel fluorescence (MCF) of JY and MCA/HHD cells stained with HLA-A2 specific antibody BB7.2 (fig 4.6.1B) was compared. The MCF of stained JY cells was nearly 20 fold over that of MCA/HHD. Therefore the differences in IFN $\gamma$  induction between JY and MCA/HHD cells in this experiment were not due to increased expression of HLA-A2 molecules in the latter.



**Fig 4.6.1 Avidity of HHD transgenic mice derived CA9<sub>254-262</sub> specific CTL is greater for CA9<sub>254-262</sub> peptide pulsed MCA/HHD than JY targets** (A) IFN $\gamma$  ELISpot assay of HHD derived CA9<sub>254-262</sub> specific CTL used as effector cells and co-incubated with CA9<sub>254-262</sub> peptide pulsed MCA/HHD tumour cells (squares), JY cells (circles). Effector : target ratio was 1:10. (*mean counts in duplicate wells shown, with 1 SD error bars*). (B) FACS histogram plots of JY (red) and MCA/HHD (green) cells stained with HLA-A2 specific antibody BB7.2 and secondary anti-mouse PE antibody. HLA-A2 negative cell line LG2 (blue) and isotype control (solid purple) in comparison.

The IFN $\gamma$  ELIspot data was supported by a  $^{51}\text{Cr}$  release cytotoxicity assay, which also confirmed the reduced recognition of peptide pulsed JY targets compared with MCA/HHD targets (fig 4.6.2). Thus for a given level of peptide pulsed on target cells, there was more specific lysis of MCA/HHD targets than JY.

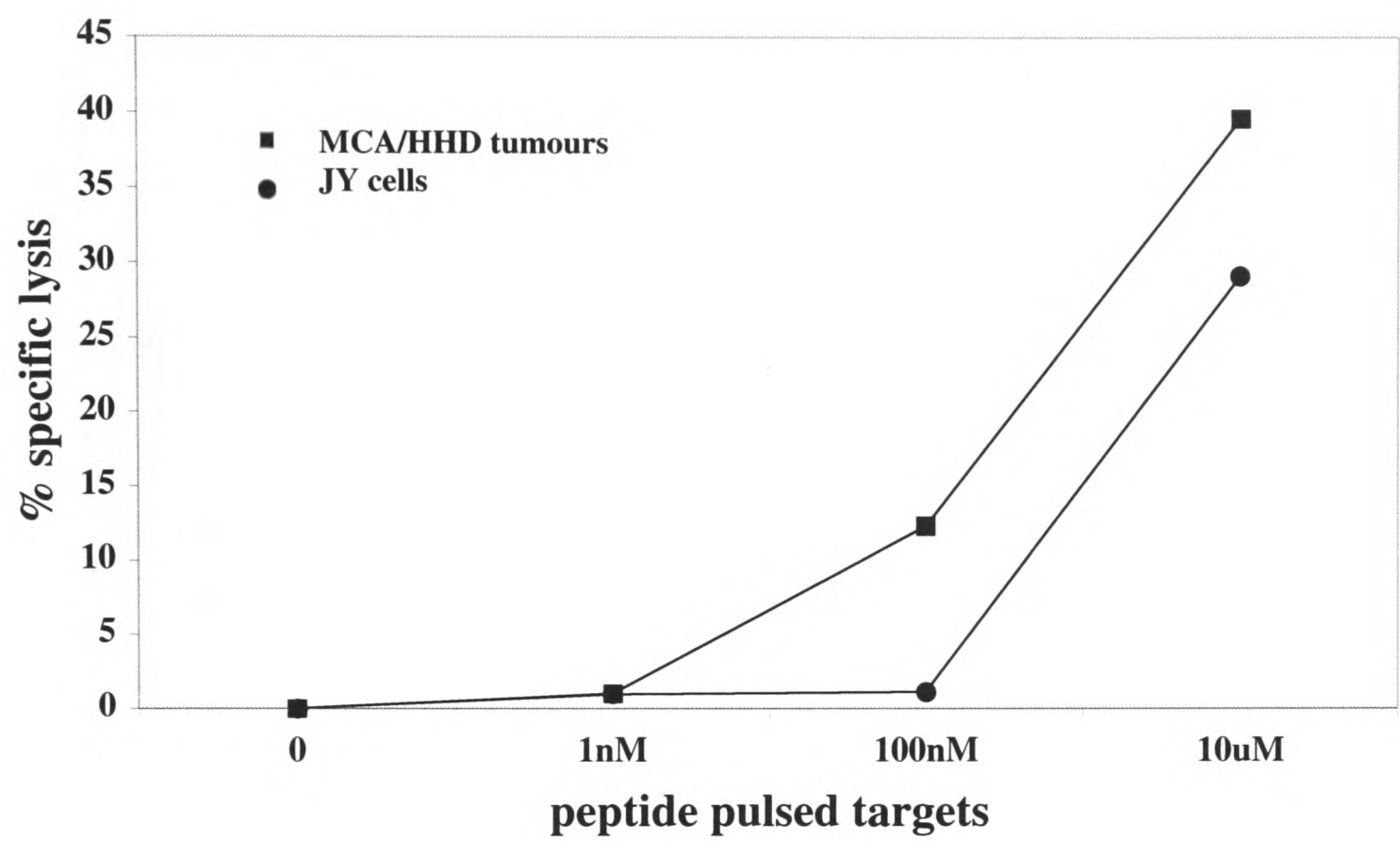
In summary, although CD8 independent tetramer staining of specific CTL indicates a higher avidity phenotype, the data suggests that induction of effector function, including IFN $\gamma$  release and cytotoxicity, are more effective with CD8 engagement.

#### **4.7 HHD transgenic mice derived CA9<sub>254-262</sub> specific CTL fail to recognize endogenously processed CA9 antigen on virally transduced JY cells**

To confirm the effect of a lack of CD8 binding by endogenous CA9 expressing human cells on subsequent TCR downstream events, JY cells were pulsed with 10 $\mu\text{M}$  CA9<sub>254-262</sub> peptide as well as infected with the recombinant vaccinia virus encoding full length CA9 for use as targets in IFN $\gamma$  ELIspot and  $^{51}\text{Cr}$  release cytotoxicity assays. To exclude any defects in the endogenous pathway of MHC I presentation, a recombinant vaccinia virus encoding only the minigene for CA9<sub>254-262</sub> was generated and used to infect JY targets (see Materials and methods). For controls, unpulsed JY cells, and JY cells infected with recombinant vaccinia virus encoding for irrelevant antigens (the Mel 3 polyepitope construct) were used.

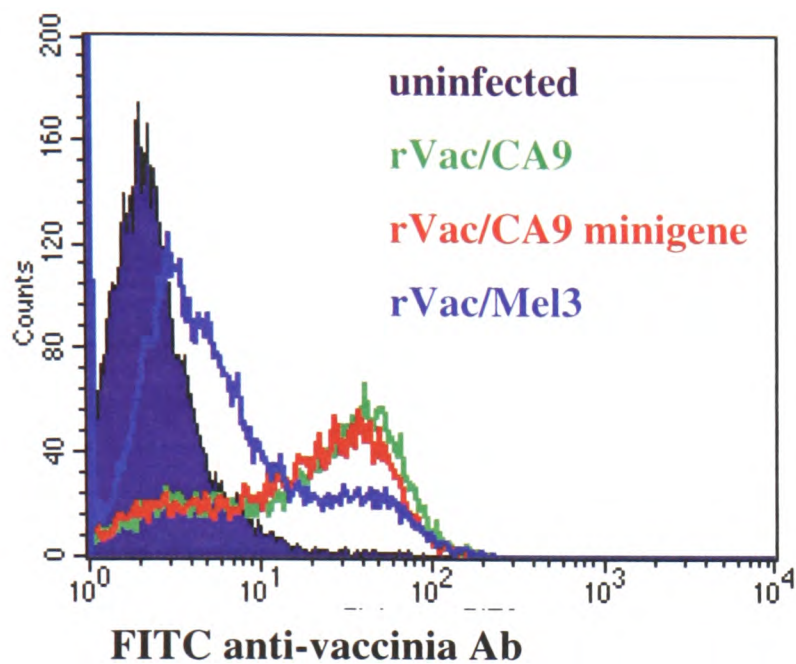
Initially, the expression of CA9 protein on transduced JY cells was checked with CA9 specific M75 antibody staining and analysed by flow cytometry (fig 4.7.1). An IFN $\gamma$



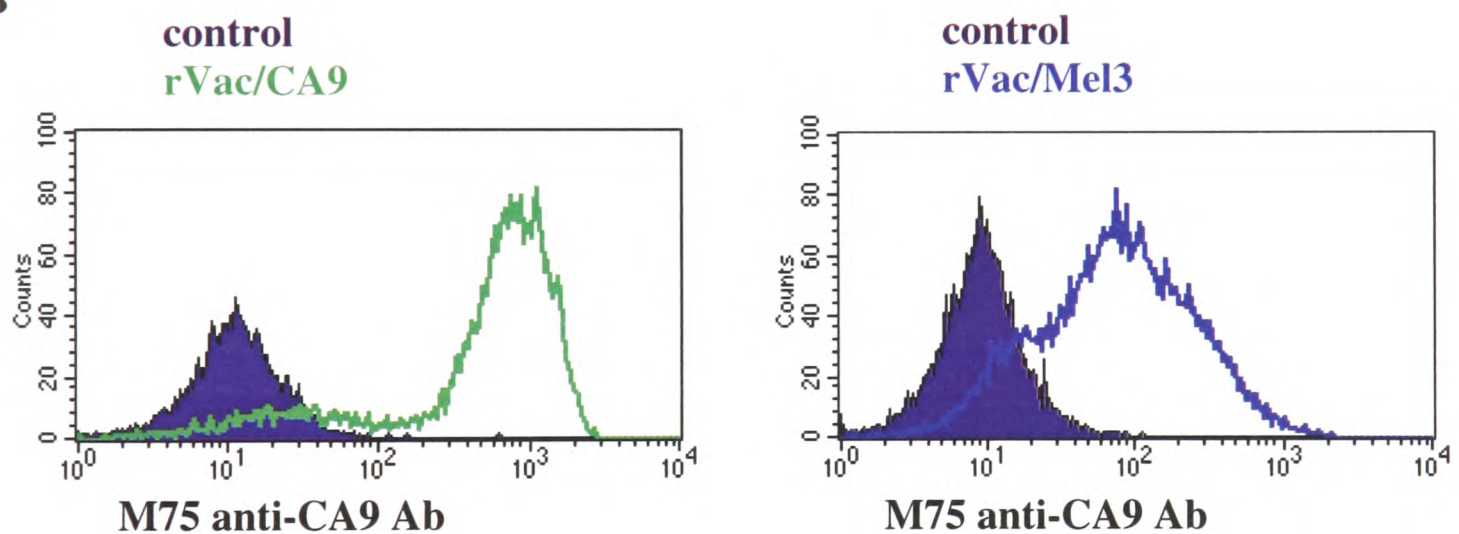


**Fig 4.6.2 Specific lysis of CA9<sub>254-262</sub> peptide pulsed targets by HHD derived CA9<sub>254-262</sub> specific CTL is greater for MCA/HHD than JY targets.** <sup>51</sup>Cr release assay with HHD derived CA9<sub>254-262</sub> specific CTL used as effector cells and co-incubated with <sup>51</sup>Cr loaded CA9<sub>254-262</sub> peptide pulsed MCA/HHD tumour cells (squares) or JY cells (circles). Effector : target ratio was 10:1.

A



B

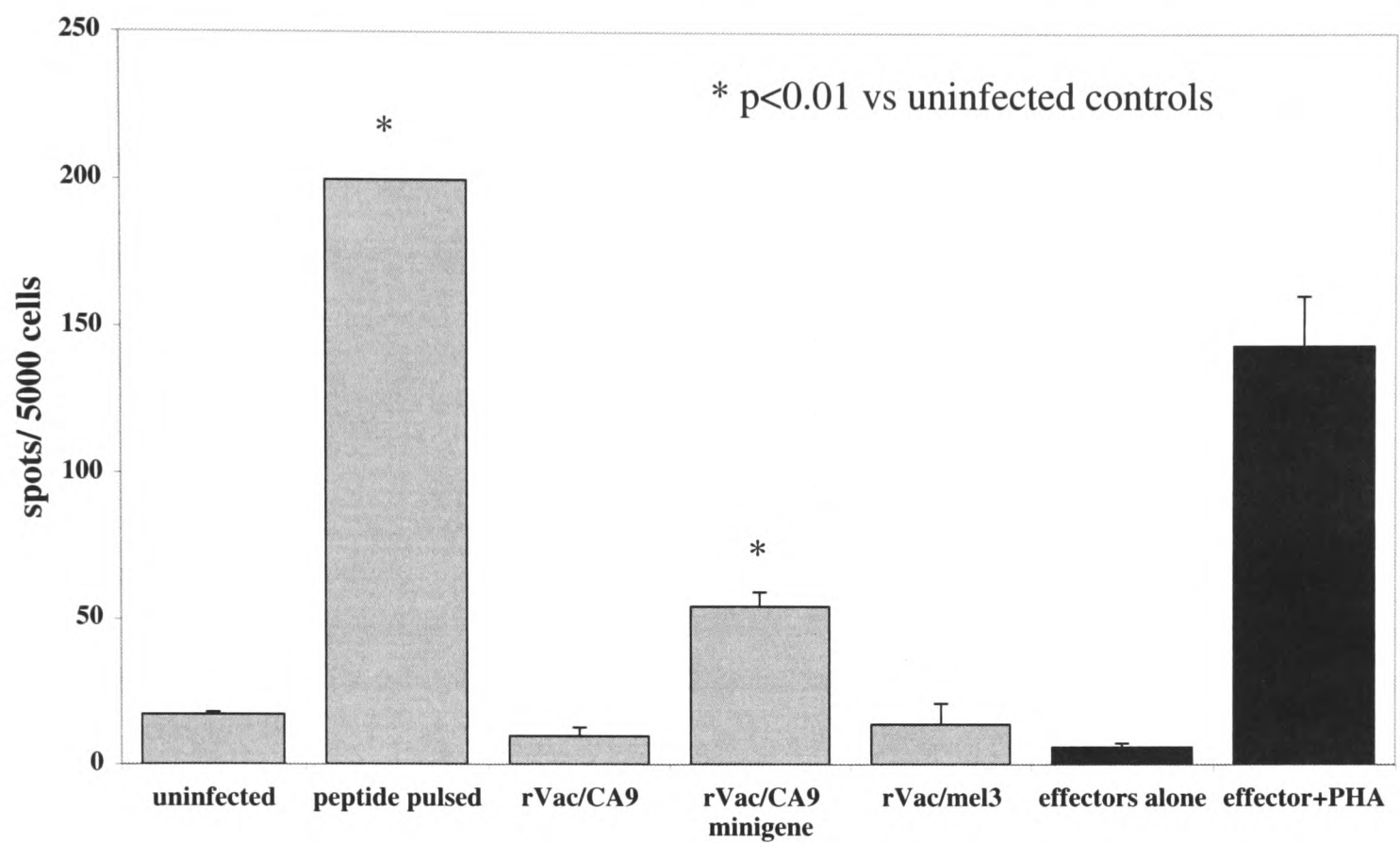


**Fig 4.7.1 JY target cells infected with rVac/CA9 express CA9 protein detectable by M75 antibody.** FACS histogram plots of JY cells infected with rVac/CA9, rVac/CA9<sub>254-262</sub> and rVac/Mel3. (A) JY cells stained with vaccinia specific antibody. Cells were fixed, permeabilized and stained with FITC conjugated anti-vaccinia antibody. (*Uninfected JY cells in solid purple. Green = rVac/CA9, red = rVac/CA9<sub>254-262</sub>, blue = rVac/Mel3*) (B) JY cells infected with rVac/CA9 (left) and rVac/Mel3 (right), and stained with primary CA9 specific antibody M75, and secondary anti-mouse PE antibody. (*Solid purple = control without primary Ab, green = rVac/CA9, blue = rVac/Mel3*)

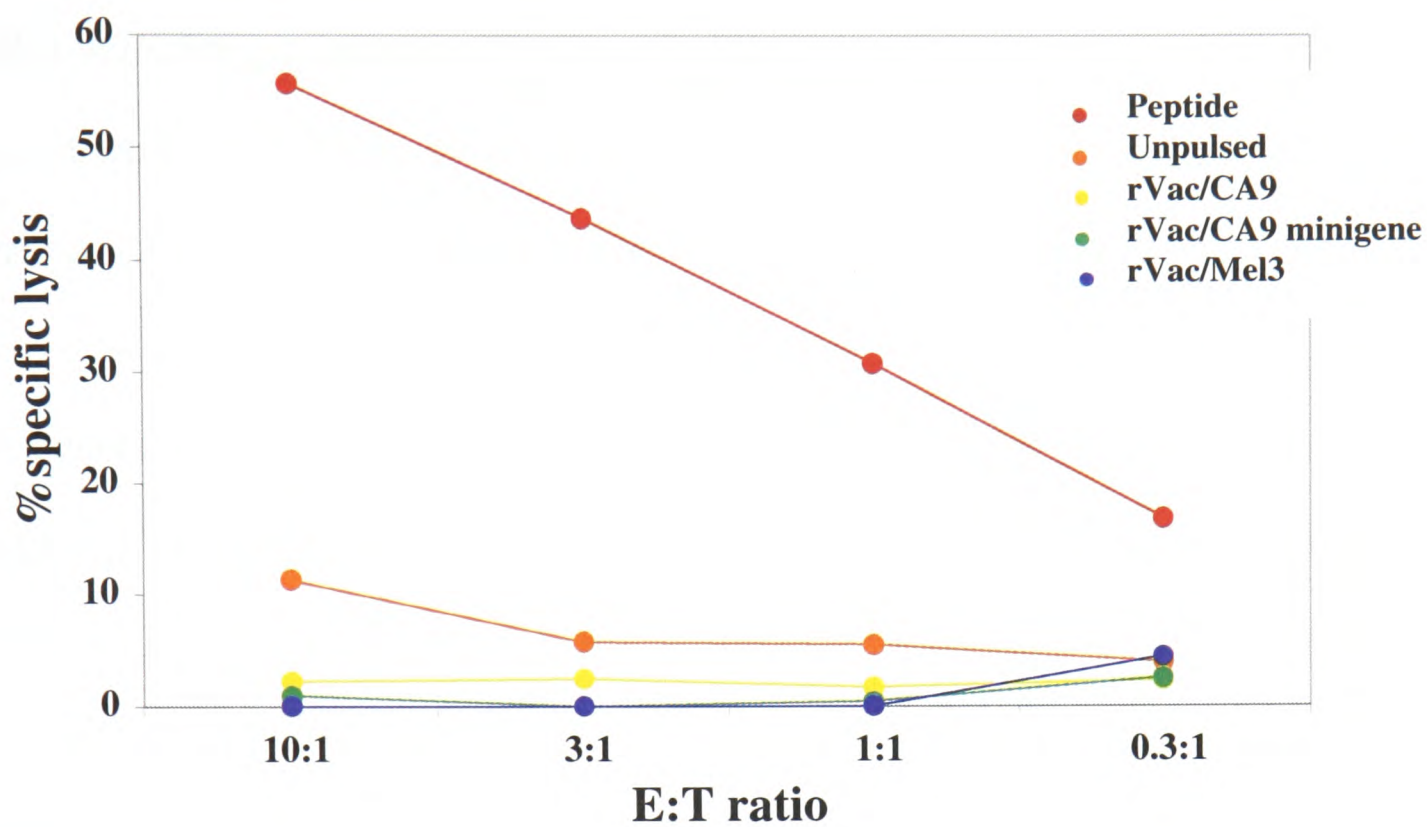
ELIspot assay was used as a readout for HLA-A2/CA9<sub>254-262</sub> specific CTL effectors following recognition of CA9<sub>254-262</sub> peptide expressing targets. These effectors were co-incubated with peptide-pulsed and virus transduced JY targets. The results demonstrate robust release of IFN $\gamma$  by HLA-A2/CA9<sub>254-262</sub> specific CTL when combined with peptide-pulsed JY targets (fig 4.7.2). Recombinant vaccinia encoding for full length CA9 (rVac/CA9) infected JY cells failed to induce IFN $\gamma$  release; while in contrast, recombinant vaccinia encoding for the CA9<sub>254-262</sub> minigene (rVac/CA9 minigene) infected targets elicited a detectable IFN $\gamma$  response.

The IFN $\gamma$  ELIspot data was confirmed with a <sup>51</sup>Cr release cytotoxicity assay (fig 4.7.3). The results showed that only JY targets that had been loaded exogenously with peptide were lysed. Interestingly, neither rVac/CA9 nor rVac/CA9<sub>254-262</sub> minigene infected targets were lysed. The data suggest that while rVac/CA9 minigene transduced targets were able to endogenously present the CA9<sub>254-262</sub> peptide, other differences could account for the lack of cytotoxicity despite a positive IFN $\gamma$  ELIspot response.

In summary, HLA-A2/CA9<sub>254-262</sub> specific CTL recognise JY targets exogenously pulsed with peptide, but not endogenously presented CA9 through viral transduction. This suggests ineffective processing and presentation of the cognate peptide, possibly through immunodominance by other epitopes, since transduction by recombinant virus for the minigene reversed this.



**Fig 4.7.2 HHD derived CA9<sub>254-262</sub> specific CTL effectors recognise JY target cells pulsed with CA9<sub>254-262</sub> peptide or rVac/CA9<sub>254-262</sub> minigene infected.** IFN $\gamma$  ELISPOT assay showing number of IFN $\gamma$  positive spots from HHD derived CA9<sub>254-262</sub> specific CTL effectors when co-incubated with JY target cells (grey bars) which were uninfected, CA9<sub>254-262</sub> peptide (10 $\mu$ M) pulsed or virus infected as indicated. Control wells showing IFN $\gamma$  spots from CA9<sub>254-262</sub> specific CTL effectors alone without JY targets, and blasted with polyclonal mitogen PHA (black bars). (*mean counts in duplicate wells shown, with 1 SD error bars. \* Comparison of means using one way ANOVA with Dunnetts multiple comparisons test*)



**Fig 4.7.3 HHD derived CA9<sub>254-262</sub> specific CTL effectors specifically lyse JY target cells pulsed with CA9<sub>254-262</sub> peptide.** A <sup>51</sup>Cr release assay was used to demonstrate specific lysis of JY target cells by HHD derived CA9<sub>254-262</sub> specific CTL effectors. JY targets were pulsed with 10 μM CA9<sub>254-262</sub> peptide (red), unpulsed (orange), or infected with rVac/CA9 (yellow), rVac/CA9<sub>254-262</sub> minigene (green), rVac/Mel3 (blue) . (*E:T* = effector: target )

## 4.8 Discussion

This chapter was primarily concerned with the generation of transgenic HHD derived HLA-A2/CA9<sub>254-262</sub> specific CTL to confirm the specificity of novel A2K<sup>b</sup> and A2/CA9<sub>254-262</sub> tetramers, as well as the identification of high avidity CTL for the recognition of endogenously expressed CA9 on human cells.

A comparison of the various vaccination protocols employed (Table 4.3) reveal that where rVac/CA9 was used, no specific responses were detected, except in one of twelve mice of cohort C. In contrast, robust responses were obtained with DNA priming followed by peptide pulsed BMDC boost (cohort D). The most likely explanation lies in the failure of natural processing and presentation of the cognate CA9 peptide in those regimes with no responses. However, the positive response generated in the one mouse vaccinated with peptide-IFA priming and rVac/CA9 boosting suggests that processing and presentation of CA9<sub>254-262</sub> peptide is possible, albeit uncommon. Apart from the lack of processing and presenting the correct peptide, immunodominance by epitopes from CA9 or vaccinia virus could account for the failure of rVac/CA9 immunisation. Palmowski *et al* (Palmowski et al., 2002) showed that immunodominance within a polyepitope string could be bypassed by separating individual epitopes onto different vectors. This was suggested by the data where rVac/CA9 minigene was used to transduce JY targets resulting in a positive IFN $\gamma$  ELIspot response (fig 4.7.2). Alternatively, it is possible that correct processing and presentation of the CA9<sub>254-262</sub> peptide occurred, but was at a level insufficient to induce a response detectable by tetramers.

The data from the HHD mice vaccinations corroborate published reports from Choi *et al* (Choi et al., 2002), demonstrating the utility of novel A2K<sup>b</sup> tetramers in transgenic murine models in detecting specific CTL responses *ex vivo*. In fig 4.4, A2K<sup>b</sup> tetramers detected a larger proportion of tetramer+/CD8+ cells encompassing a range of tetramer binding affinities. In contrast, CD8 independent CTL responses as revealed by A2 tetramer staining has been reported as a phenomenon associated with high avidity CTL (Choi et al., 2003; Pittet et al., 2003).

When HHD derived CA9<sub>254-262</sub> specific CTL were used against naturally presented CA9 peptides in a human tumour cell line RCC4, they failed to effect specific lysis. However, this was reversed through exogenous loading with 10  $\mu$ M peptide. This pattern was similar to the findings with rVac/CA9 infected and peptide pulsed JY targets. The HHD derived CA9<sub>254-262</sub> specific CTL were able to recognize cognate peptide (10 $\mu$ M) pulsed on JY cells, but not rVac/CA9 infected targets. These target cells were successfully transduced by virus since they expressed CA9 protein on the surface as detected by M75 staining. The data suggest that the endogenously presented peptide/MHC - TCR avidity was not sufficient to result in HHD derived CA9<sub>254-262</sub> specific CTL activation or killing of human targets. In contrast, the avidity of the CTL for pMHC as assayed by peptide titration on HHD derived murine targets was in the range of up to 100 nM peptide (fig 4.6.2), which was within the 500 nM threshold for TCR activation by peptide titration as reported by Sette *et al* (Sette et al., 1994). The lack of CD8 binding in human JY targets increased the threshold for cytotoxicity to 10  $\mu$ M peptide. Therefore the loss of avidity of



HHD derived CA9<sub>254-262</sub> specific CTL in the absence of CD8 ligation on RCC4 targets could have accounted for the lack of TCR activation and killing. Alternatively, the failure of correct processing and presentation of the cognate peptide by RCC4 cells is a possibility.

The physiological function of CD8 co-receptor binding continues to be debated. Four possible roles for the CD8 co-receptor have been proposed: as a cell adhesion molecule (Luescher et al., 1995), an alternative signal transduction pathway through its association with p56<sup>lck</sup> (Purbhoo et al., 2001), as a stabilizer of TCR/pMHC complex (Garcia et al., 1996; Luescher et al., 1995), and through direct interaction with TCR (Doucey et al., 2003). Wyer and others (Wyer et al., 1999) failed to detect any enhancement of TCR binding by CD8 to specific peptide-MHC class I ligands on surface plasmon resonance studies. Affinity studies revealed CD8/MHC binding to be weaker than TCR/pMHC ligation (Matsui et al., 1994; Moody et al., 2001; Purbhoo et al., 2001), but that CD8 co-receptor continued to exhibit biological activity at low binding affinities (Hutchinson et al., 2003). TCR activation by monomeric and multimeric pMHC complexes have demonstrated CD8 dependence (Daniels and Jameson, 2000; Delon et al., 1998). Class I tetramer staining and TCR activation have been shown to be blocked by anti-CD8 antibodies (Campanelli et al., 2002; Daniels and Jameson, 2000; Denkberg et al., 2001), although steric hindrance by these antibodies cannot be excluded as suggested by Woolridge *et al* (Wooldridge et al., 2003). Consistent with these observations, those targets expressing CD8 binding  $\alpha 3$  domain derived from H-2D<sup>b</sup>(MCA/HHD tumours) showed higher levels of specific lysis than targets with non-binding HLA-A2  $\alpha 3$  domains



(JY cells) (fig 4.6.1A). This difference was not due to increased expression of surface HLA-A2 molecules in MCA/HHD over JY cells; in fact, human JY cells expressed nearly 20 fold more HLA-A2 complexes than MCA/HHD.

Interestingly, rVac/CA9<sub>254-262</sub> minigene infected JY cells were recognized on IFN $\gamma$  ELISPOT assay but failed to result in specific lysis (compare fig 4.5.3 with 4.5.4). The induction of peptide-MHC class I complexes by cytosolic minigene derived products is significantly increased relative to full length gene products. Porgador *et al* (Porgador *et al.*, 1997) have used antibodies specific for the K<sup>b</sup>/Ova<sub>257-264</sub> complex to reveal that approximately 3500 complexes were expressed on the surface of cells infected with rVac/OVA, compared with more than 65 000 complexes per cell with rVac expressing cytosolic minigene products. Also, as few as a single peptide-MHC complex on a target cell has been shown to be sufficient to elicit a cytotoxic T cell response (Sykulev *et al.*, 1996). Thus, the failure of rVac/CA9<sub>254-262</sub> minigene infection to elicit a CTL response cannot be explained by quantitative differences. Instead, it is more plausible that the failure of binding with the CD8 co-receptor by peptide-MHC complexes on JY targets was responsible for the partial agonist action observed. It has been shown (see Chap 5) that rVac/CA9<sub>254-262</sub> minigene infection of JY cells can result in specific cytolytic effects when targeted by human CA9<sub>254-262</sub> specific CTL, suggesting that it is indeed CD8 co-receptor binding by effector T cells that makes the difference. Xu *et al* (Xu *et al.*, 2001) demonstrated good correlation of CD8 co-receptor binding with TCR activation and cytotoxicity, with reduced activity in the absence of CD8 binding to  $\alpha$ 3 domain mutants of pMHC I complexes. Significantly, FasL mediated apoptosis of targets was intact,

suggesting that limited TCR signaling without CD8 binding could still trigger expression of preformed intracellular FasL. Esser *et al* (Esser et al., 1996) have shown the distinct signalling requirements for perforin or FasL mediated cytotoxicity, and IFN $\gamma$  expression, with the latter two mechanisms still intact in the absence of a strong TCR triggered Ca<sup>2+</sup> flux. The data suggests that partial agonist action in the absence of CD8 binding resulted in IFN $\gamma$  expression (fig 4.7.2), while specific cytotoxicity (as detected by 5 hours <sup>51</sup>Cr release assay) was not activated (fig 4.7.3).

In summary, it has been shown that CA9<sub>254-262</sub> specific CTL responses to human CA9<sub>254-262</sub> peptide could be induced in HHD transgenic mice, and specifically detected through the use of novel A2 and A2K<sup>b</sup> tetramers. These effectors could recognize foreign (human) CA9 antigen with high avidity when exogenously presented on syngeneic MHC complexes, but had reduced avidity in the absence of CD8 co-receptor binding to the  $\alpha$ 3 domain of the MHC class I molecule. Viral transduction of JY targets with rVac/CA9 encoding for the full length protein, or natural constitutive expression of CA9 by RCC4 did not result in any TCR activation. Therefore, the utility of these transgenic HHD derived CTL in studies involving human targets is limited by the failure to recognise endogenously presented antigen. It remains to be established whether the cognate peptide was properly processed and presented from full length protein in these systems. Transduction with the viral minigene for CA9<sub>254-262</sub> peptide partially restored recognition and resulted in IFN $\gamma$  expression, but not cytotoxicity as determined in a 5 hour killing assay, indicating partial agonist action through the TCR in the absence of CD8 binding.

## Chapter 5. CA9/G250 specific T cell responses in healthy donors and renal cell cancer patients

### 5.1 Introduction

The existence of natural T cell immunity to cancer (reviewed by Nagorsen (Nagorsen et al., 2003), is implicit in the immune-surveillance hypothesis (see chapter 1). The identification of tumour antigens recognised by T cells, as well as improvements in analysing T cell responses, has made it possible to establish *ex vivo*, the natural T cell responses to tumours in un-vaccinated patients and even healthy subjects. These responses potentially form the basis of rationale vaccine designs aimed at inducing effective anti-tumour T cell responses *in vivo*.

Malignant cutaneous melanoma is the most extensively studied malignancy in tumour immunology. Spontaneous T cell responses to the melanoma differentiation antigen melanA/ MART-1 have been reported in peripheral blood as well as tumour infiltrated-lymph node derived lymphocytes (Dhodapkar et al., 2000; Griffioen et al., 2001; Lee et al., 1999b; Pittet et al., 1999). Unique among all the tumour antigens to date, T cell responses to Melan-A/MART-1 (HLA-A2 restricted peptide 26-35 EAAGIGILTV) in healthy donors have been reported, ranging from 8–60 % of individuals studied. Although the actual ligands responsible for positive and negative thymic selection of this epitope are unknown, Pittet *et al* (Pittet et al., 2002) have suggested that abundant thymic presentation of cross-reactive sequences could bias towards such an unusually high frequency in the periphery. Dutoit *et al* reported that the high frequency of Melan-A

multimer+ T cells could be explained by the existence of largely cross-reactive subsets of naive CD8+ T cells displaying multiple specificities. (Dutoit et al., 2002). With this exception, such frequent T cell responses against a tumour antigen are rarely found in healthy individuals. Other less frequent T cell responses to tumour antigens in melanoma patients have been reported, such as to NY-ESO-1 (Valmori et al., 2000), MAGE-A10 (Valmori et al., 2001), CAMEL (Rimoldi et al., 2000) and survivin (Andersen et al., 2001), a member of the inhibitor of apoptosis protein gene family.

Rentzsch *et al* (Rentzsch et al., 2003) reported T cell responses in 54% of HLA-A\*0201 breast cancer patients to a range of tumour antigens, including MUC-1, Her-2/neu, CEA, NY-ESO-1 and SSX-2. Interestingly, Nagorsen and colleagues (Nagorsen et al., 2000) detected natural T cell responses against tumour antigens Ep-CAM, Her-2/neu and CEA, in approximately half of colorectal cancer patients studied, with responses confined to those patients with lymph node or distant metastases but not organ confined disease. Hoffman *et al* (Hoffmann et al., 2002) analysed blood samples from patients with head and neck cancers using wild type p53 specific tetramers, and found 23 out of 30 with p53-specific T cell responses. Recently, Korangy and others (Korangy et al., 2004) reported spontaneous tumour specific humoral and cellular immune responses to NY-ESO-1 in hepatocellular carcinoma patients, with responses correlating with NY-ESO-1 expression on the tumours.

RCC are thought to be immunogenic because of reported incidences of biological responses to cytokine based therapies, and also rare spontaneous tumour regressions (as

high as 6.6% in the placebo arm of an IFN $\gamma$  treatment for metastatic RCC trial (Gleave et al., 1998). Natural tumour specific T cell responses have been reported from TIL (Finke et al., 1994; Schendel et al., 1993) and PBL (Brouwenstijn et al., 1996). A host of tumour antigens have been identified in relation to RCC specific T cell responses, including RAGE, gp75 (Neumann et al., 1998), PRAME (Kessler et al., 2001), and SART (Kawagoe et al., 2000; Shintaku et al., 2000). As described previously, over 90% of RCC patients bear the clear cell variant of RCC, a tumour phenotype that has been associated with the von-Hippel Lindau (VHL) mutation and constitutive expression of CA9, with none in normal tissue. Based on these evidence, it was reasonable to hypothesize that CA9 could be a valid tumour associated antigen for T cell directed therapy.

It is speculative whether CA9 specific T cell responses are centrally deleted in the thymus, considering that CA9 is in effect a self-antigen. An emerging body of evidence suggests that CA9 specific T cell precursors do exist in the periphery. Tso et al has reported the use of a novel CA9-GMCSF fusion protein that is immunostimulatory for peripheral blood mononuclear cells (PBMC) ex vivo, resulting in CA9 specific CD8 $^{+}$  T cell mediated antitumour responses against autologous tumours (Tso et al., 2001). Autologous dendritic cells transfected with total renal tumour RNA have been shown to elicit T cell responses to CA9 in a Phase I trial (Su et al., 2003). Uemura reported at the 94<sup>th</sup> AACR annual conference (Uemura, 2003) that HLA-A24 restricted 9-mer peptide vaccines for CA9 were administered subcutaneously in 5 patients with disseminated RCC, with 3 patients displaying antigen specific T cell mediated cytotoxicity. More

recently, Mukouyama and colleagues reported that PBMC derived monocytes transduced with a recombinant adenovirus encoding CA9 could generate CTL in vitro capable of lysing CA9 expressing cancer cells (Mukouyama et al., 2004).

Work by Vissers et al (Vissers et al., 1999) suggests that some HLA-A2/CA9-derived peptide responses are inducible in healthy HLA-A2 human donor PBMC in vitro. Following on from their murine data, three peptides were found to be immunogenic in humans. Of these, only peptide CA9<sub>254-262</sub> was both immunogenic and endogenously processed/ presented in human BLM melanoma cells. Interestingly, the group extended their findings to a HLA-DR restricted epitope that overlapped CA9<sub>254-262</sub>, and was recognised by CD4<sup>+</sup> T cells (Vissers et al., 2002). This peptide was therefore thought to be potentially of clinical importance to A2/CA9 peptide specific vaccine design.

In this chapter, the natural T cell responses to a hypoxia-regulated tumour antigen CA9, in healthy donors as well as renal cell cancer (RCC) patients were characterised. Healthy donor buffy coats and blood specimens from RCC patients were processed as described. Peripheral blood lymphocytes (PBL) were isolated, restimulated with CA9 derived peptides and analysed in vitro. The avidity and functionality of these CA9 specific CTL were analysed, and correlated with the expression of CA9 on the tumour specimens, as well as with clinical data.

## 5.2 In vitro priming of PBL from a healthy HLA-A2 donor with CA9<sub>254-262</sub> peptide

A HLA-A2/CA9<sub>254-262</sub> peptide specific response from a healthy donor was induced by utilizing an in vitro priming technique previously described (Salio et al., 2001), using autologous monocyte-derived DC pulsed with the relevant peptide. To determine the phenotype of GM-CSF and IL4 differentiated CD14<sup>+</sup> monocytes, staining with CD14 and CD1a antibodies was performed and analysed by flow cytometry. The FACS plots showed a transformation from CD14<sup>+</sup>/CD1a<sup>-</sup> phenotype to CD14<sup>-</sup>/CD1a<sup>+</sup> dendritic cells (fig 5.2.1). The immature dendritic cells thus generated were matured as described with CD40 ligand expressing J558 cells, with analysis of activation markers CD80, 83 and 86 showing significant upregulation, signifying a mature dendritic cell phenotype (fig 5.2.1).

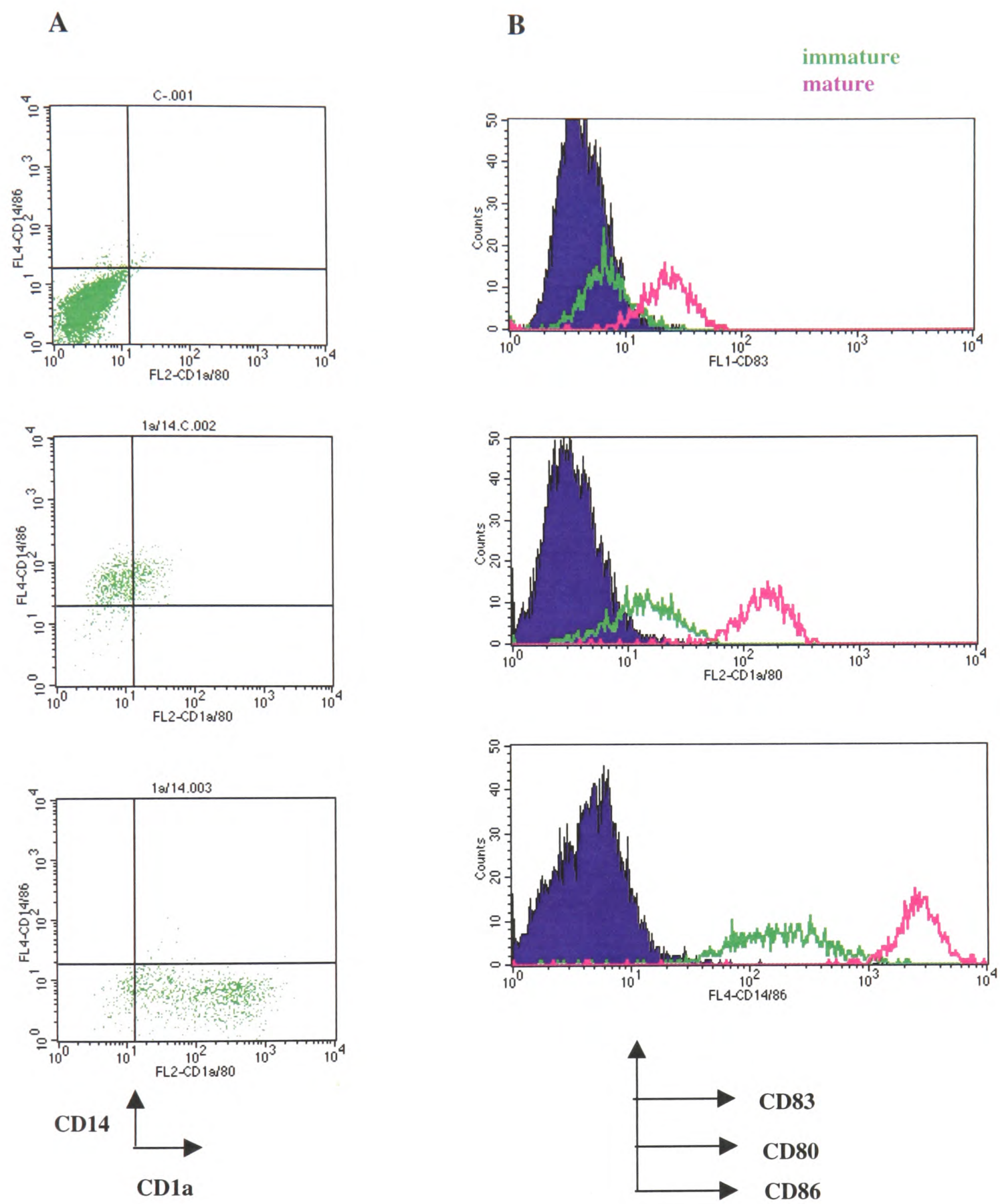
Mature peptide pulsed DC were incubated with responder autologous CD14<sup>-</sup> PBMC. Following in vitro priming and restimulation with irradiated peptide pulsed autologous CD14<sup>-</sup> PBMC, PBL were stained with A2/CA9<sub>254-262</sub> tetramers and analysed by flow cytometry. As controls, PBL from the same donor were primed and restimulated against another tumour antigen Melan-A (HLA-A2 restricted epitope ELAGIGILTV). Fig 5.2.2 shows successful induction of A2/CA9<sub>254-262</sub> specific CTL (0.2% tetramer +/ CD8<sup>+</sup> cells) 10 days after restimulation with A2/CA9<sub>254-262</sub> peptide. No A2/CA9<sub>254-262</sub> responses were seen in the Melan-A stimulated culture.



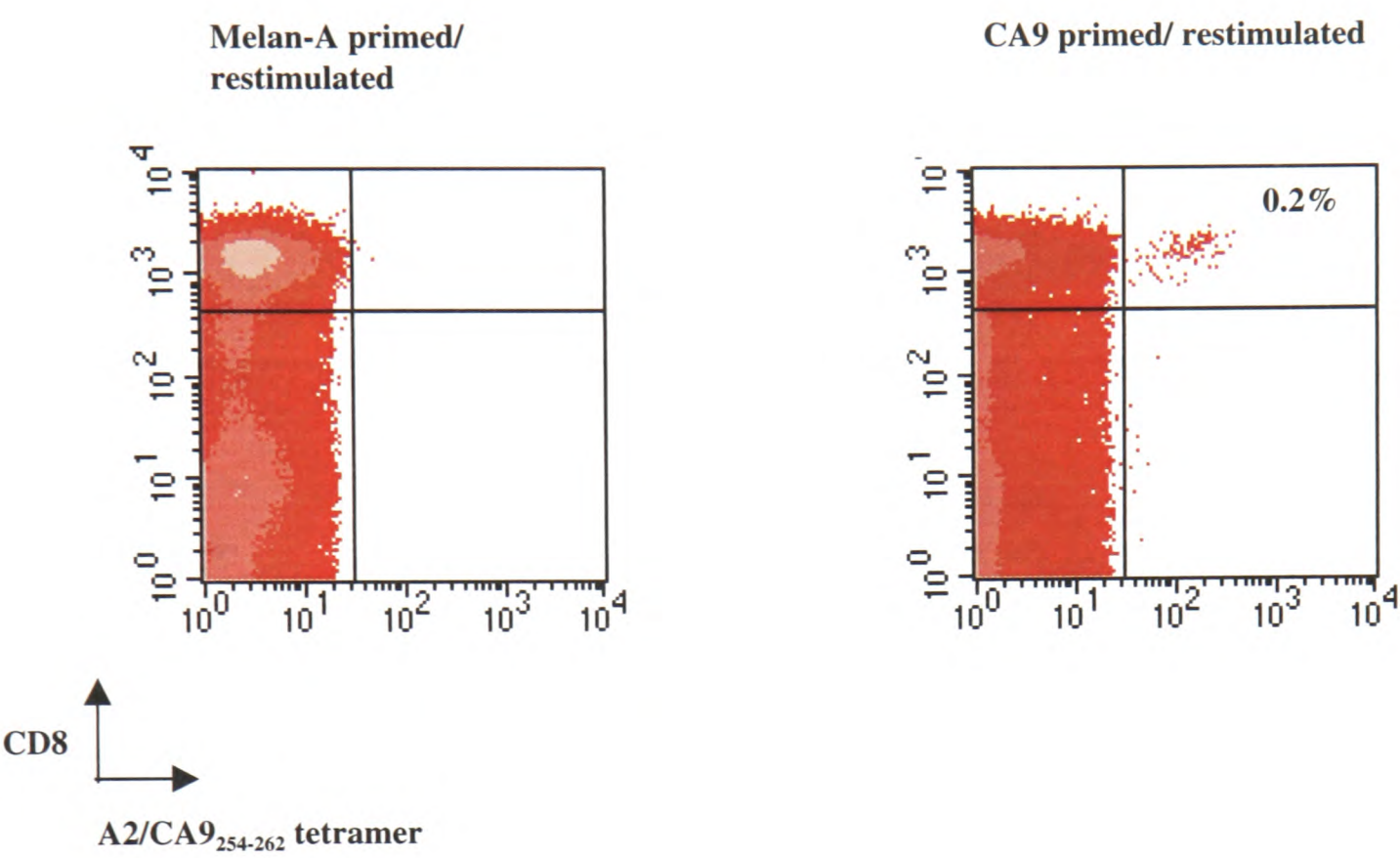
### 5.3 Tetramer-based fluorescence activated cell sorting of HLA-A2/CA9<sub>254-262</sub> peptide specific CTL lines

To further enrich the CTL specificity for the A2/CA9<sub>254-262</sub> epitope in vitro, cell sorting by FACS using A2/CA9<sub>254-262</sub> tetramer stained PBL from a healthy HLA-A2 donor from section 5.2 was performed as described in Materials/Methods. HLA-A2/CA9<sub>254-262</sub> specific T cell lines were successfully expanded and subsequently analysed by tetramer staining and flow cytometry (fig 5.3). For comparison, the same CTL line was stained with a tetramer for an irrelevant peptide, in this case ELA. There was an enrichment to over 95% tetramer +/ CD8 + cells following FACS and in vitro expansion. The following functional assays were performed on this enriched population of A2/CA9<sub>254-262</sub> specific CTL line.

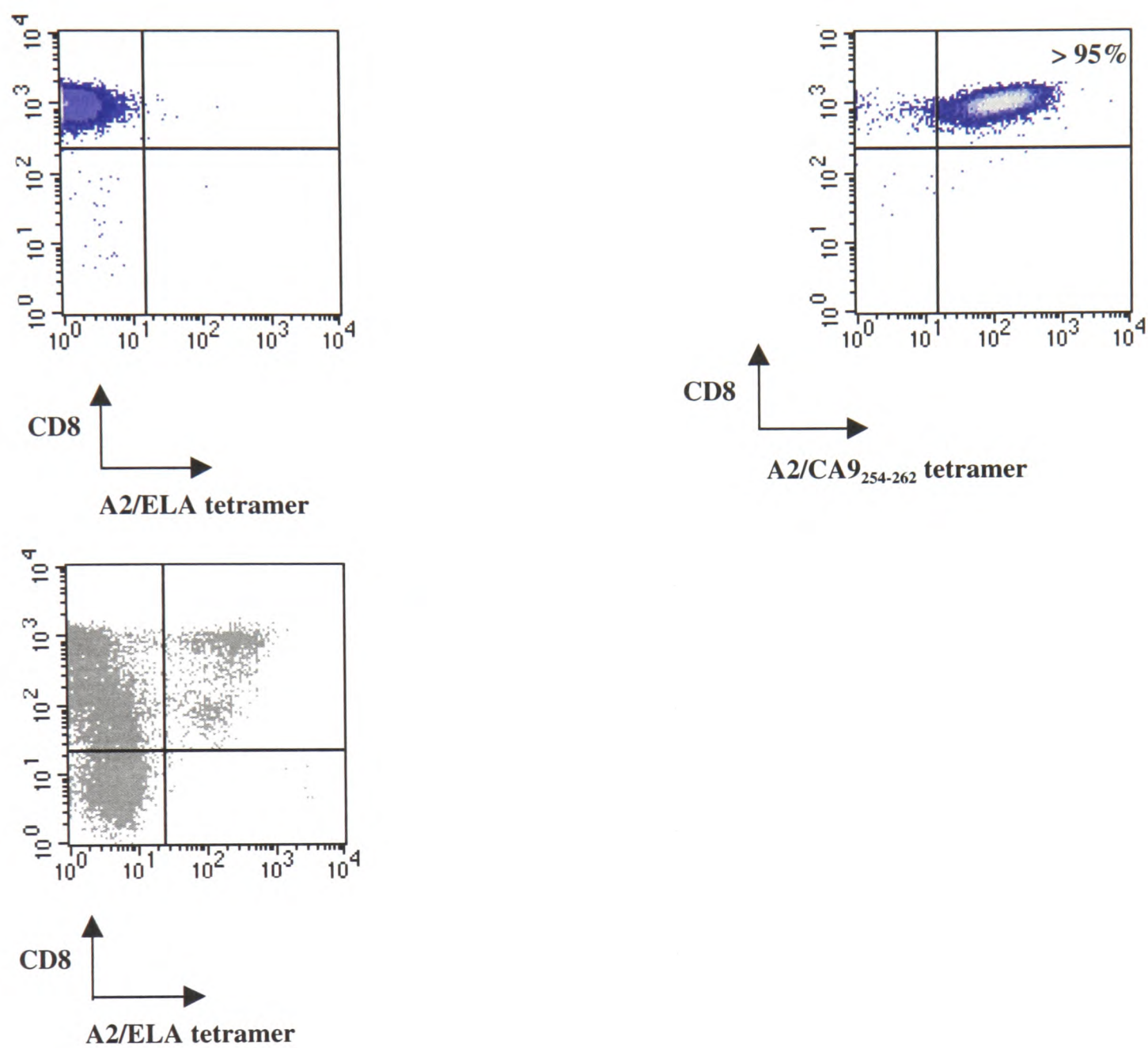




**Fig 5.2.1 Generation of mature monocyte-derived DC from CD14+ PBMC using GM-CSF and IL4, with CD40 ligand maturation signal. (A) FACS plots showing phenotype of DC derived from GM-CSF and IL4 pulsed CD14+ PBMC (lowest) compared with unpulsed PBMC (middle), and unstained DC (upper). (B) Histograms showing upregulation of activation markers CD80, 83, 86; in CD40 ligand activated mature DC (pink), compared with immature DC (green) and unstained mature DC (solid purple).**



**Fig 5.2.2 In vitro priming and restimulation induces a A2/CA9<sub>254-262</sub> specific response in PBL from a healthy HLA-A2 donor.** FACS plots of PBL derived from a healthy HLA-A2 donor, in vitro primed and restimulated with A2/CA9<sub>254-262</sub> peptide or Melan-A derived HLA-A2 restricted ELA peptide. Staining with A2/CA9<sub>254-262</sub> tetramer and acquired for FACS analysis on day 10 post-restimulation. Figure in upper right quadrant shows percentage tetramer+/ CD8+ cells.



**Fig 5.3 A2/CA9<sub>254-262</sub> specific CTL line enriched by tetramer based FACS and in vitro restimulation.** FACS plots from PBL of a healthy HLA-A2 donor from fig 5.2.2 after enrichment by A2/CA9<sub>254-262</sub> tetramer based FACS, and in vitro restimulation with anti-CD3/CD28 antibodies. Staining with A2/CA9<sub>254-262</sub> tetramer or control tetramer (A2/ELA tetramer) and acquired for FACS analysis on day 21 post-sorting/ restimulation (blue plots). A2/ELA tetramer staining of Melan-A peptide restimulated unsorted PBL shown in comparison (grey plot). Figure in upper right quadrant shows percentage tetramer +/- CD8 + cells.

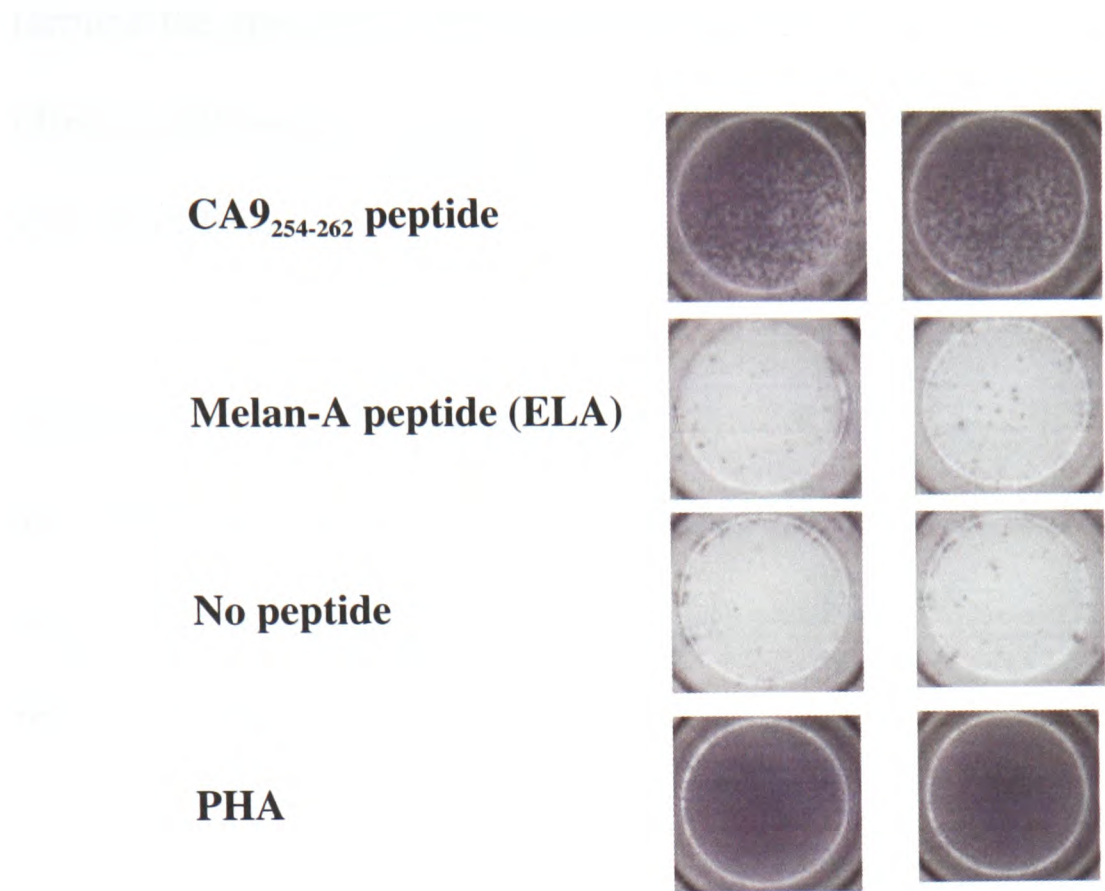
#### **5.4 A2/CA9<sub>254-262</sub> CTL line from a healthy donor express IFN $\gamma$ upon recognition of cognate peptide**

To assess if the A2/CA9<sub>254-262</sub> CTL line derived from a healthy donor was capable of expressing IFN $\gamma$  in response to cognate peptide, an IFN $\gamma$  based ELIspot assay was performed. For comparison, the same cells were pulsed with an irrelevant peptide ELA.

Fig 5.4 shows that expression of IFN $\gamma$  in CA9<sub>254-262</sub> peptide pulsed CTL was peptide specific, since there was no recognition of ELA peptide, and no IFN $\gamma$  expression in the absence of peptide.

Further analysis (section 5.5 – 5.9) of the cytotoxic capability of this A2/CA9<sub>254-262</sub> specific CTL line was performed using a standard 5 hour <sup>51</sup>Cr release assay as described in Materials/Methods.





**Fig 5.4 A2/CA9<sub>254-262</sub> CTL express IFN $\gamma$  on recognition of cognate peptide.** IFN $\gamma$  ELISPOT assay of A2/CA9<sub>254-262</sub> CTL upon restimulation with CA9<sub>254-262</sub> peptide or Melan-A derived peptide ELA. No peptide and PHA restimulated wells as controls. Each well assayed in duplicate.

### **5.5 A2/CA9<sub>254-262</sub> CTL recognize and specifically lyse CA9<sub>254-262</sub> peptide pulsed JY targets, but are of low avidity**

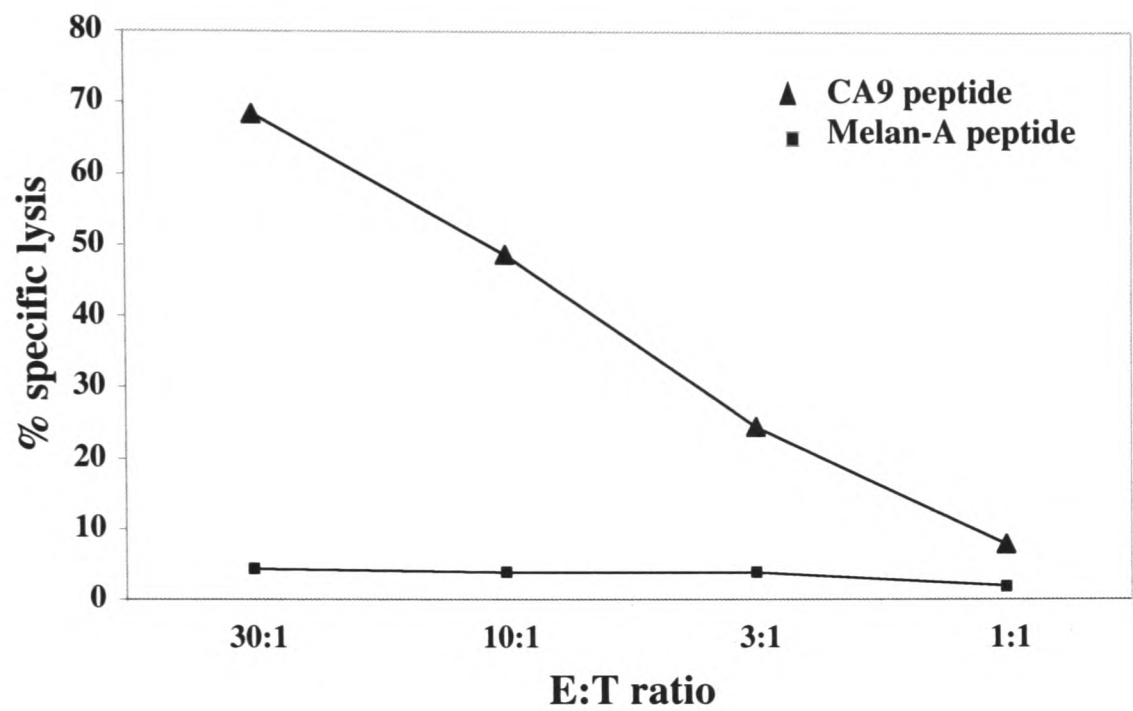
To determine the specificity and avidity of healthy human donor derived A2/CA9<sub>254-262</sub> CTL, effector cells were co-incubated with JY targets labelled with <sup>51</sup>Cr and pulsed with peptide as described.

The results demonstrate that recognition and lysis of CA9<sub>254-262</sub> peptide pulsed targets was specific, since there was no lysis of Melan-A peptide (ELA) pulsed targets (fig 5.5A). The avidity of these CTL was low, since targets pulsed with less than 1μM peptide failed to be recognized (fig 5.5B).

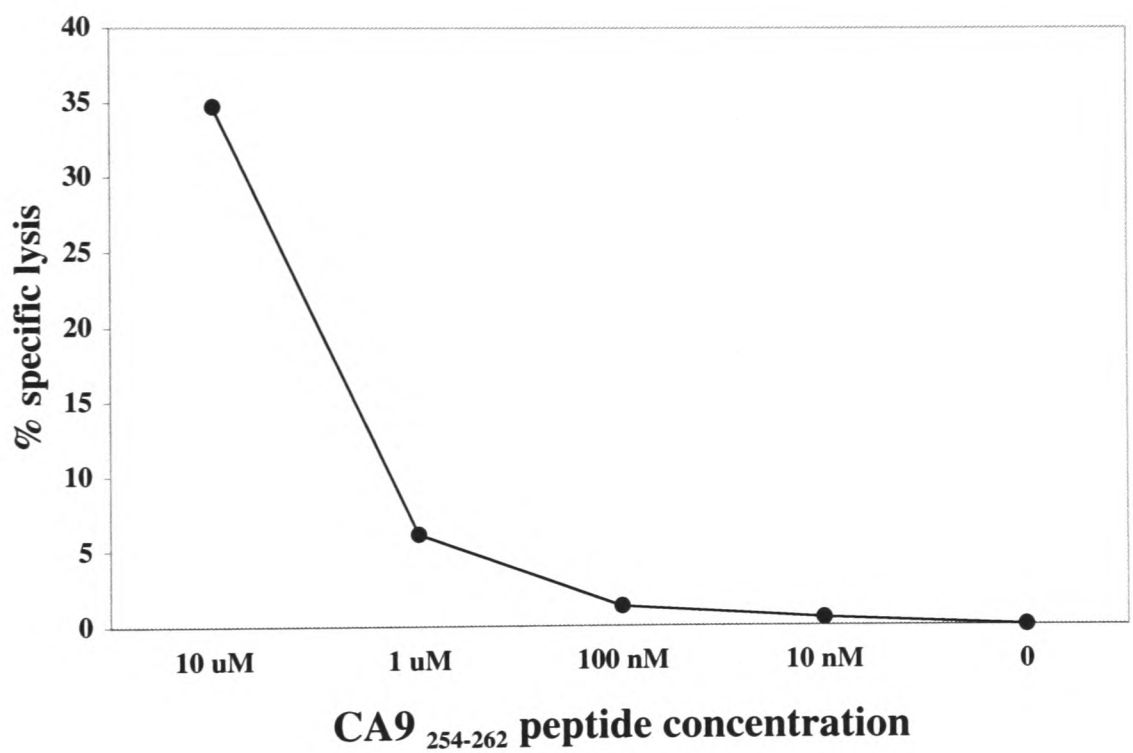
### **5.6 A2/CA9<sub>254-262</sub> CTL fail to recognize endogenous CA9 antigen in rVac/CA9 transduced JY targets, but recognise peptide derived from the CA9<sub>254-262</sub> minigene**

The recognition by A2/CA9<sub>254-262</sub> CTL for endogenously presented peptides derived from rVac transduced JY cells encoding full length protein or CA9<sub>254-262</sub> minigene was analysed. To show that JY targets were successfully transduced by recombinant vaccinia, analysis for expression of CA9 and reaction with anti-vaccinia antibody was performed as in fig 4.7.1 (data not shown). Fig 5.6 shows that specific lysis was restricted to peptide pulsed (10 and 1μM) and rVac/CA9<sub>254-262</sub> minigene transduced targets. There was no recognition of rVac/CA9 or control wild type vaccinia infected cells.

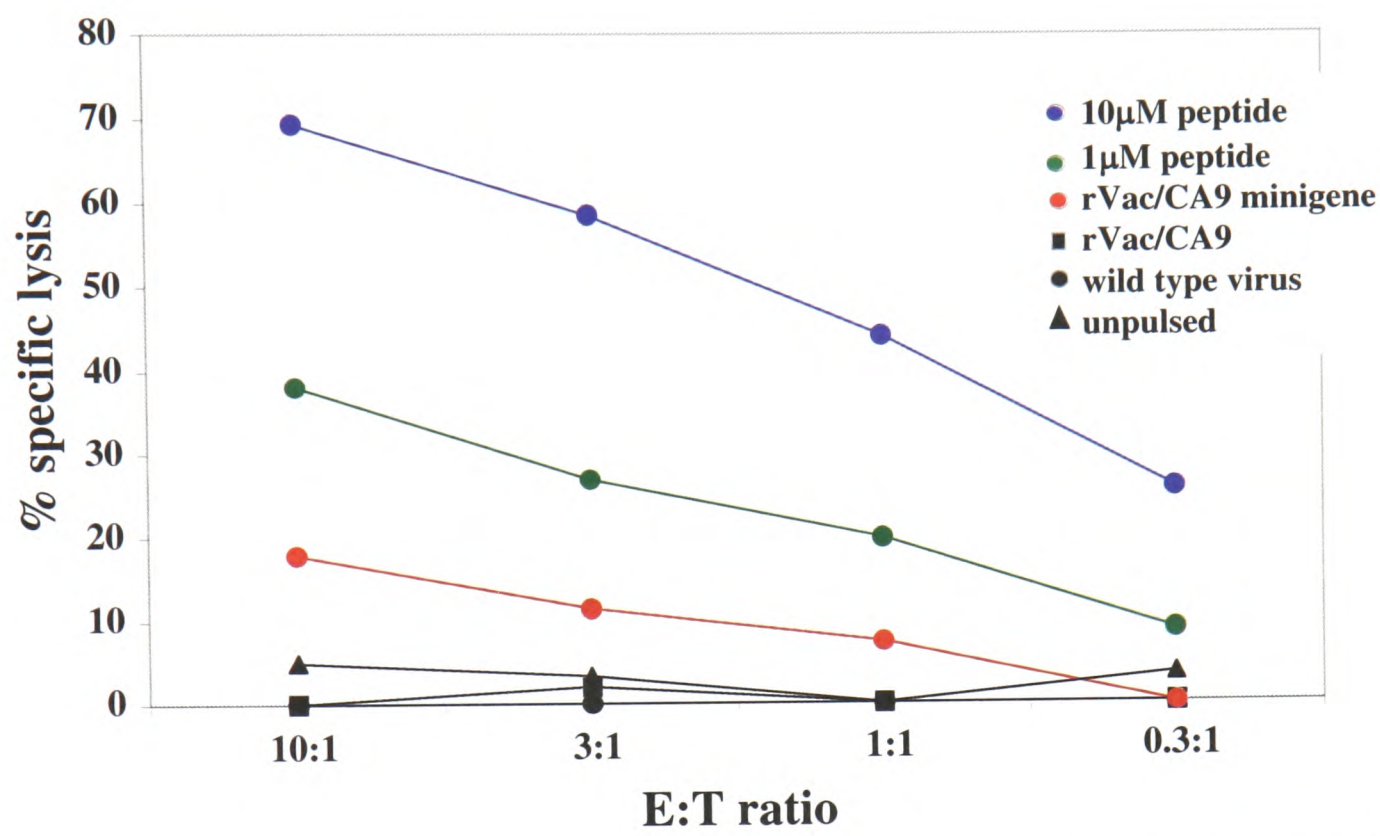
A



B



**Fig 5.5 A2/CA9<sub>254-262</sub> specific CTL recognise and lyse CA9<sub>254-262</sub> peptide pulsed targets with low avidity.** (A) <sup>51</sup>Cr release assay was used to demonstrate specific lysis of CA9<sub>254-262</sub> peptide pulsed JY targets (triangles) by A2/CA9<sub>254-262</sub> specific CTL , with Melan-A peptide (ELA) pulsed targets as controls (squares). All peptides used at 10 μM concentration. (*E:T = effector: target* ) (B) Similar assay was used to demonstrate specific lysis of peptide pulsed JY targets by A2/CA9<sub>254-262</sub> specific CTL in a peptide-titrated fashion. In this experiment, effector: target ratio was fixed at 10:1



**Fig 5.6 A2/CA9<sub>254-262</sub> CTL fail to recognize endogenous CA9 antigen in rVac/CA9 transduced JY targets, but recognise CA9<sub>254-262</sub> peptide derived from the minigene.** <sup>51</sup>Cr release assay to demonstrate recognition and specific lysis of JY target cells transduced with rVac/CA9<sub>254-262</sub>minigene (red), but not rVac/CA9 (black squares) or control wild type vaccinia virus (black circles). In comparison, JY targets exogenously pulsed with 10μM (blue) and 1 μM (green) CA9<sub>254-262</sub> peptide, or left unpulsed (black triangles) were used. (*E:T* = effector: target)



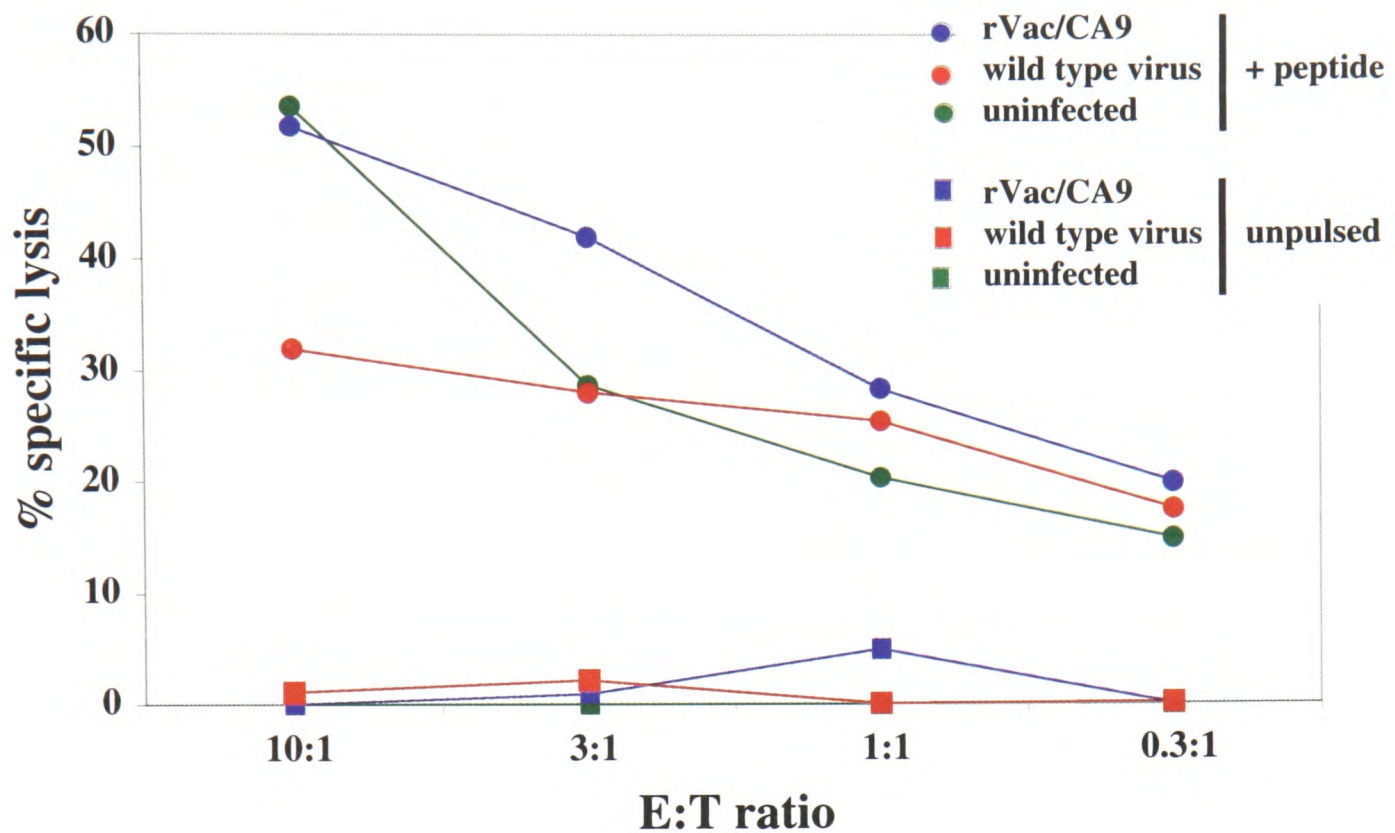
### **5.7 Expression of CA9 on JY cells does not inhibit recognition of other peptide-MHC complexes.**

Since cell surface stability of pMHC is sensitive to pH, and membrane-expressed CA9 is believed to play a role in acidification of the extracellular environment, it was hypothesised that the specific surface expression of CA9 on JY cells could inhibit recognition of pMHC I by its TCR ligand.

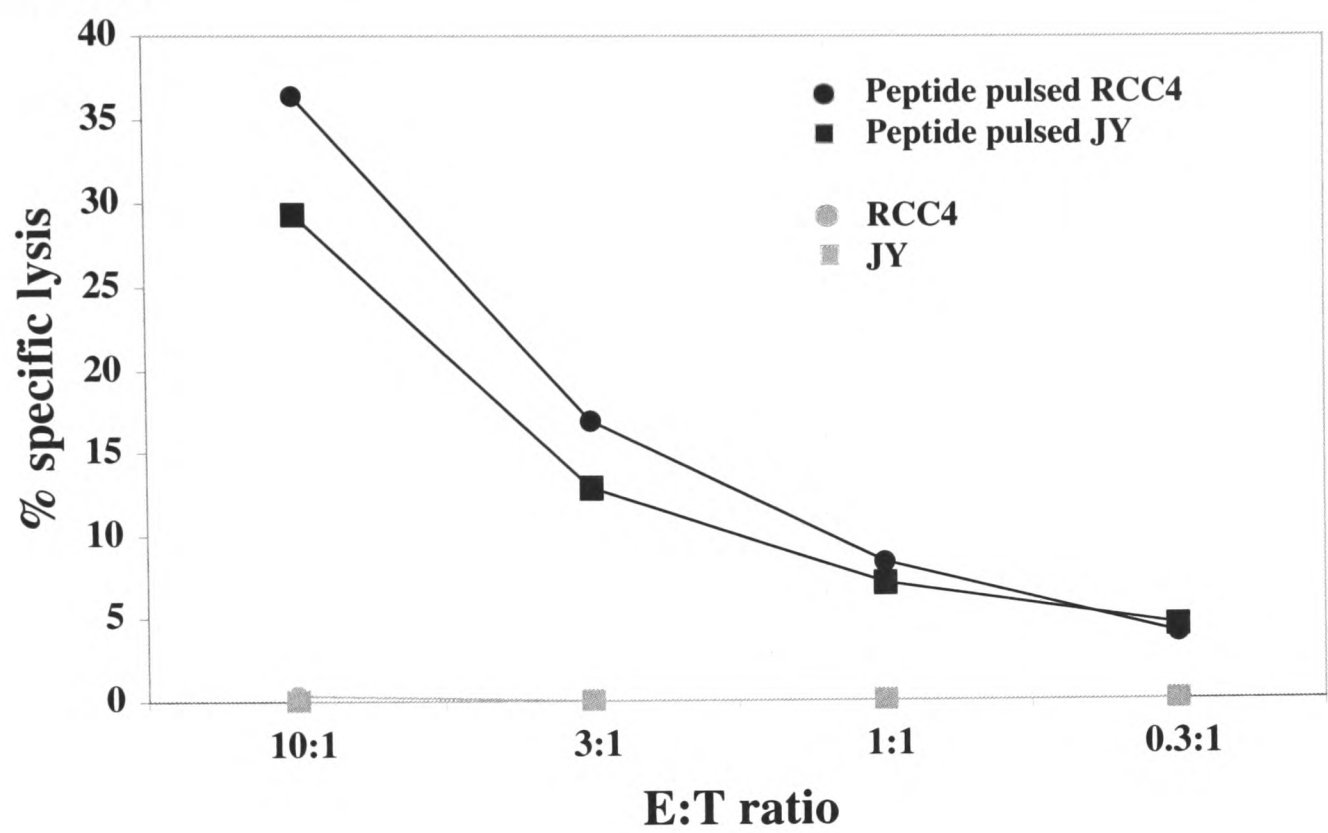
To test this hypothesis, JY cells were infected with rVac/CA9 and surface CA9 expression ascertained as before. These were then left unpulsed or pulsed with NY-ESO<sub>154-165</sub> peptide at 10  $\mu$ M for use as targets. As controls, we used wild type vaccinia virus infected JY cells with and without NY-ESO<sub>154-165</sub> peptide loading. A human NY-ESO CTL clone specific for NY-ESO<sub>154-165</sub> was used as effector cells. The results in figure 5.7 show that all peptide pulsed targets were equally recognised and lysed, regardless of expression of CA9 on the cell surface.

### **5.8 A2/CA9<sub>254-262</sub> CTL fail to recognize endogenous CA9 antigen in CA9 expressing RCC line (RCC4)**

To determine if this failure of recognition of endogenous CA9 extended to naturally expressing CA9 antigen presenting cells (APC), a previously characterised renal tumour line RCC4 (fig 4.5A) was used as a target. Fig 5.8 demonstrates that RCC4 cells alone were not lysed by A2/CA9<sub>254-262</sub> CTL, unless exogenously pulsed with 10  $\mu$ M CA9<sub>254-262</sub> peptide. JY targets pulsed with the same peptide or left unpulsed were used in comparison, which showed lysis of peptide-pulsed but not unpulsed targets. Therefore,



**Fig 5.7 Surface CA9 expression does not inhibit A2/NY-ESO<sub>154-165</sub> specific CTL recognition of NY-ESO<sub>154-165</sub> peptide pulsed JY targets.** <sup>51</sup>Cr release assay to demonstrate recognition and specific lysis of JY target cells bearing exogenously loaded 10  $\mu$ M NY-ESO<sub>154-165</sub> peptide (all circles) by A2/NY-ESO<sub>154-165</sub> specific CTL, versus unpulsed targets (all squares). JY cells were left uninfected (green), transduced by rVac/CA9 (blue) or control wild type vaccinia (red). (*E:T* = effector: target)



**Fig 5.8 A2/CA9<sub>254-262</sub> specific CTL fail to recognise CA9 expressing renal tumour line RCC4.** <sup>51</sup>Cr release assay to demonstrate recognition and specific lysis of RCC4 target cells exogenously loaded with 10 µM CA9<sub>254-262</sub> peptide (black circles) but not naturally CA9 expressing RCC4 (grey circles). For comparison, JY cells were used as unpulsed targets (grey squares), or 10µM peptide loaded (black squares). (*E:T* = *effector: target*)

A2/CA9<sub>254-262</sub> CTL were able to recognise exogenous peptide but not when endogenously presented on RCC4 tumour cells.

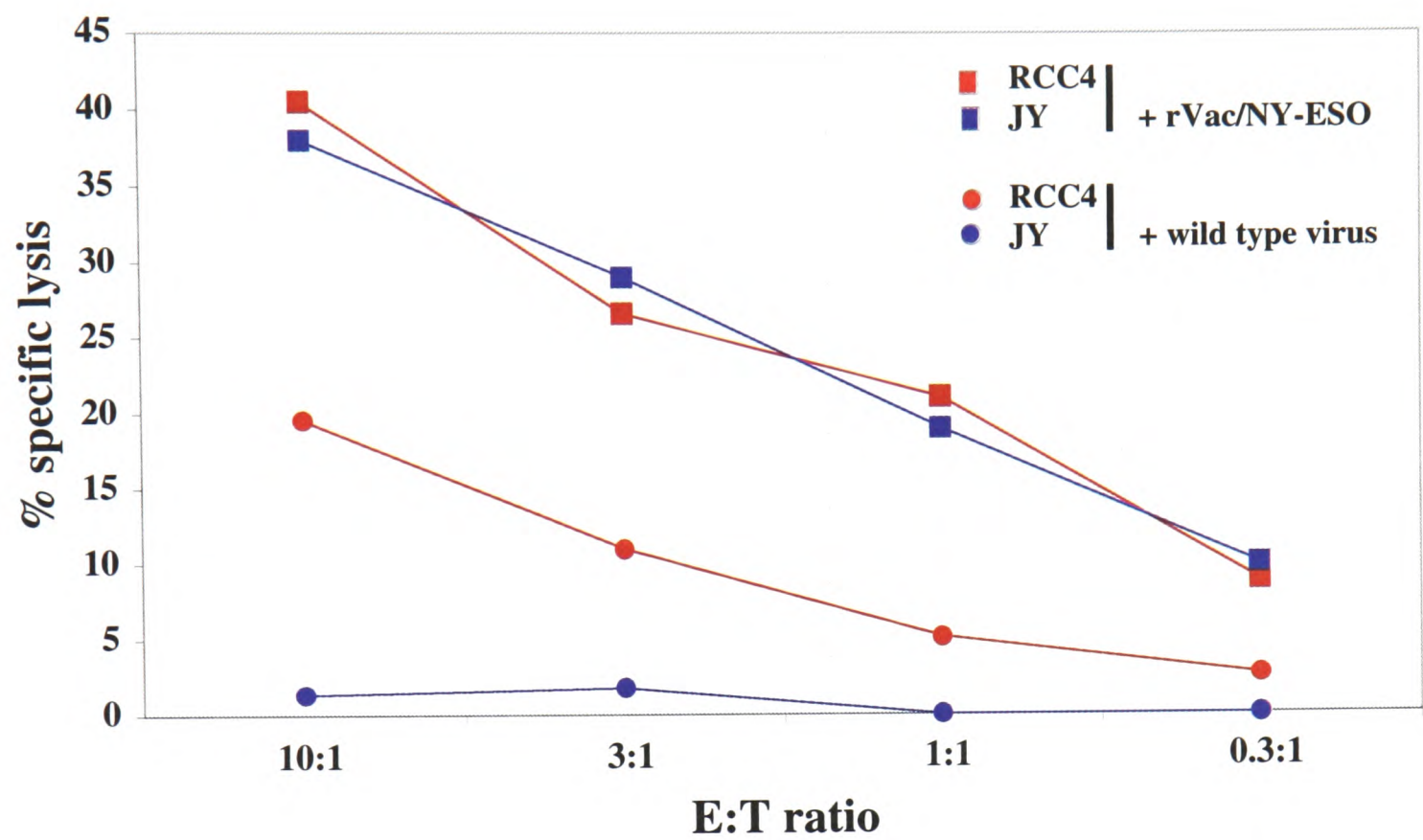
### **5.9 NY-ESO<sub>157-165</sub> specific CTL lyse JY and RCC4 targets when transduced by rVac/ NY-ESO**

To determine if the failure of specific lysis of JY and RCC4 targets was due to a generalised defect in antigen processing and presentation on MHC I, JY and RCC4 targets were infected with rVac encoding for the full length tumour antigen NY-ESO, and used NY-ESO<sub>154-165</sub> specific CTL clones as effectors.

Fig 5.9 shows that RCC4 cells, like JY controls, process and present NY-ESO antigen following rVac/NY-ESO infection as shown by lysis with NY-ESO<sub>154-165</sub> specific CTL, in comparison with control wild type vaccinia infected targets.

### **5.10 Natural T cell responses to CA9 in a cohort of RCC patients versus healthy controls**

Vissers *et al* (Vissers et al., 1999) have published data from HLA-A\*0201 healthy human donors and identified A2 restricted CA9 responses induced by four peptides. To date, there have been no published data on such responses in RCC patients. Since 90% of RCC are of the clear cell variant, which constitutively expresses membrane bound CA9 as a result of the VHL mutation, it was reasoned that natural T cell immunity to CA9 expressing tumour cells could be elicited in recall assays in such patients.



**Fig 5.9 NY-ESO<sub>154-165</sub> specific CTL recognise and lyse JY and RCC4 targets transduced by rVac/NY-ESO.** <sup>51</sup>Cr release assay to demonstrate recognition and specific lysis of JY and RCC4 target cells infected with rVac/NY-ESO (blue and red squares respectively) or control wild type vaccinia virus (blue and red circles). (*E:T* = effector: target)

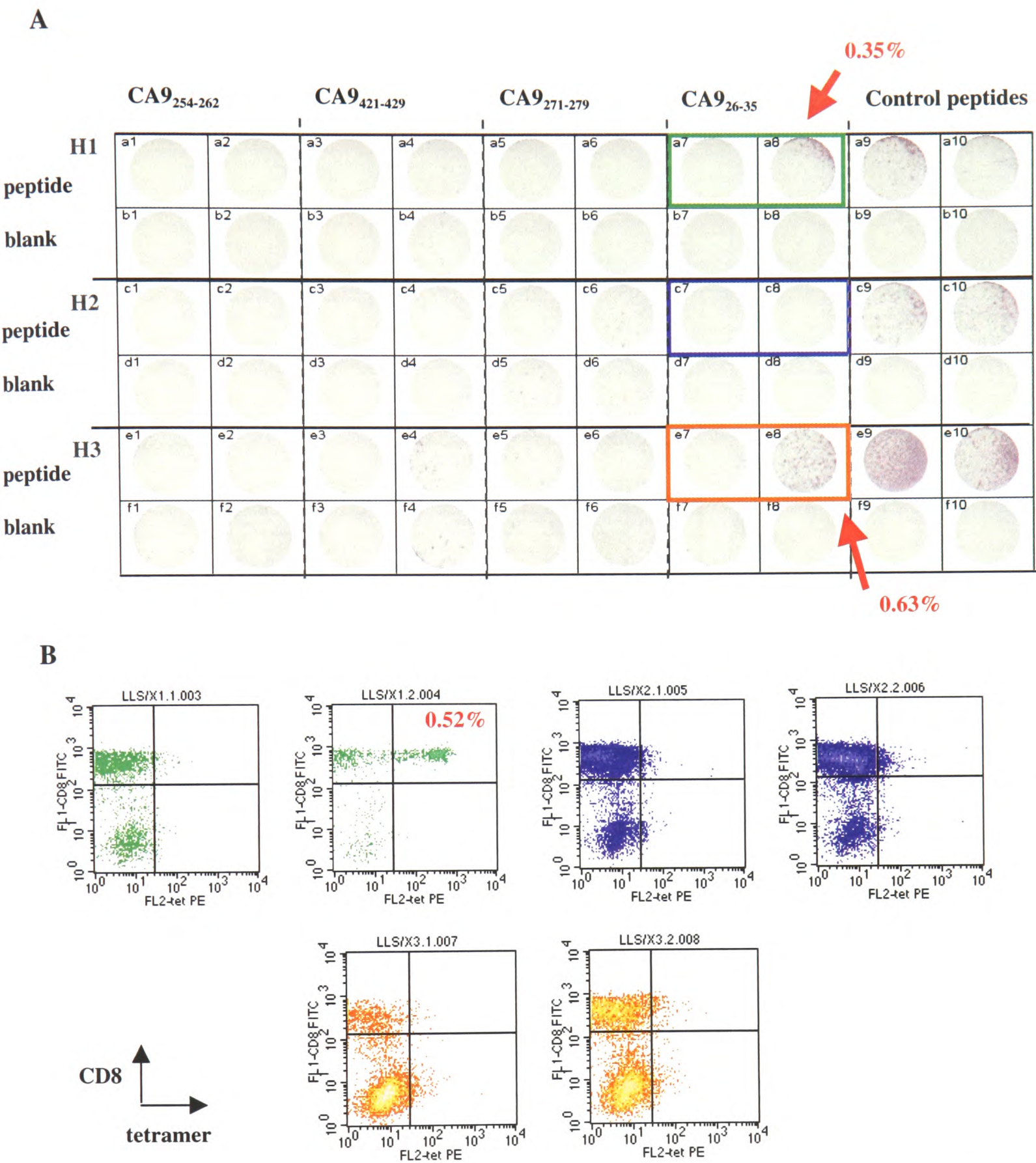
PBMC from HLA-A2 RCC patients and healthy donors were restimulated in vitro as described. Briefly, unsorted PBMC from frozen stocks were thawed and individually restimulated with 100  $\mu$ M of the four peptides mentioned above, supplemented with IL7. Cells were fed with fresh media containing IL2 from the third day, and every 3 days following. Between 10 – 12 days later, cells were restimulated with APC (.221A2 LCL) which had been pulsed with 10  $\mu$ M of the respective peptide and irradiated. These were fed again every 3 days with fresh media containing IL2. As controls for this protocol, PBMC restimulated with pooled peptides from three well characterised viral epitopes derived from influenza, Epstein-Barr and cytomegalovirus (Flu, EBV and CMV) were used.

Analysis of CA9 peptide specific responses in PBMC in vitro was performed using the IFN $\gamma$  ELISpot assay as a readout, at least 12 days after the last restimulation. Initial *ex vivo* analysis, as well as data following one round of restimulation, did not yield any detectable responses to CA9 above the background in all subjects, except to the control viral peptides (data not shown). Therefore recall assays were standardised by employing the IFN $\gamma$  ELISpot assay after three rounds of in vitro restimulation (see Materials/ methods). A response to a peptide in any individual was considered positive if the number of IFN $\gamma$  spots in either of the duplicate peptide pulsed wells were at least twice that above the background (unpulsed wells), and concurrently reactive to non-specific PHA restimulation. No differentiation was made between degrees of positivity, since the ‘all-or-none’ responses simply reflected the presence of precursor CD8 + T cells in that individual that were capable of being expanded in vitro. Novel HLA-A2/CA9 tetramers

were generated all four identified HLA-A2 restricted CA9 epitopes. To validate the findings from the IFN $\gamma$  ELISpot data, cells from the same wells were stained with corresponding tetramers and analysed by FACS. Fig 5.10.1A shows such responses from 3 representative healthy controls. In 2 wells, there were clear IFN $\gamma$  ELISpot responses to the CA9<sub>26-35</sub> peptide, of which only 1 was found to correlate with A2/CA9<sub>26-35</sub> tetramer staining (fig. 5.10.1B). Fig 5.10.2A demonstrates a similar response to the CA9<sub>26-35</sub> peptide in one RCC patient (BYW), which was correlated with tetramer staining (fig.5.10.2B). The absence of IFN $\gamma$  ELISpot responses from those wells pulsed with the CA9<sub>26-35</sub> peptide in other RCC patients correlated with the absence of A2/CA9<sub>26-35</sub> tetramer staining on FACS analysis (data not shown)

Table 5.10 summarises the data from the entire cohort of 24 consecutive HLA/A2 subjects which consisted of RCC patients prior to primary radical nephrectomy (p1-19), and those metastatic disease cases referred for biological therapy without any prior treatment (m1-5). Histology from subjects m1-5 were not available since no specimens were available. The diagnoses of RCC was therefore presumptively based on computerised tomographic scanning (CT scan) or magnetic resonance imaging (MRI). The data shows a preponderance of the clear cell variant of RCC on histology in subjects p1-19, at various stages/grades. Immunohistochemical staining of specimens for CA9 using CA9 specific antibody M75 was performed, showing diffused and strong membranous staining of the tumour cells, with none in the adjacent stroma or normal renal tissue (fig 5.10.3A/B). Interestingly, M75 staining in papillary RCC (non-clear cell RCC) showed focal staining of CA9 (fig 5.10.3C). Papillary RCC is not known to be

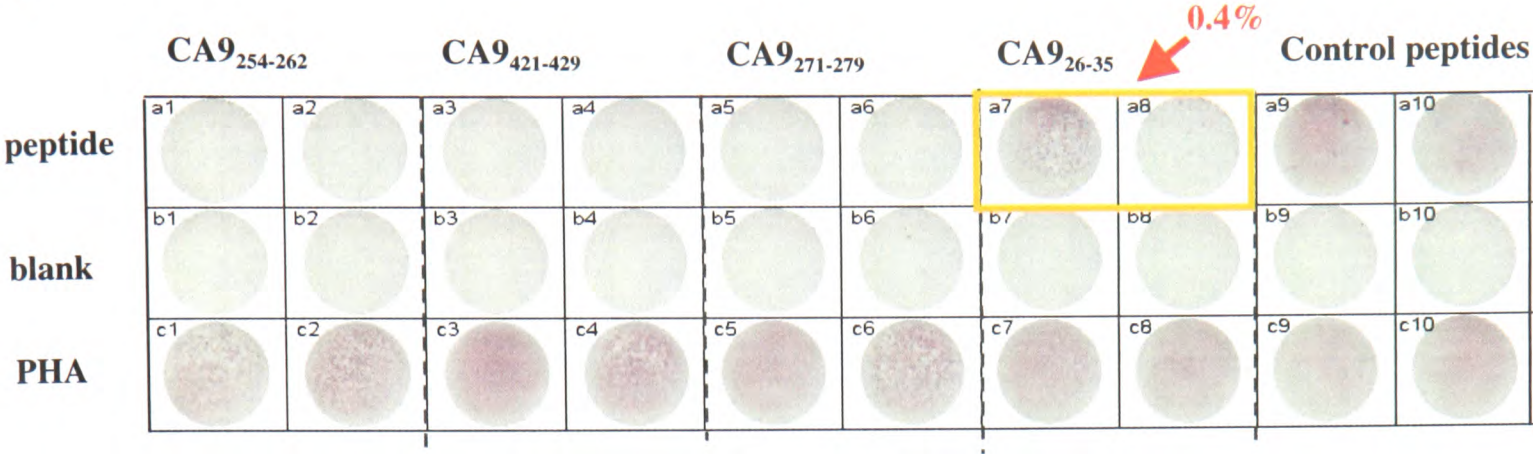




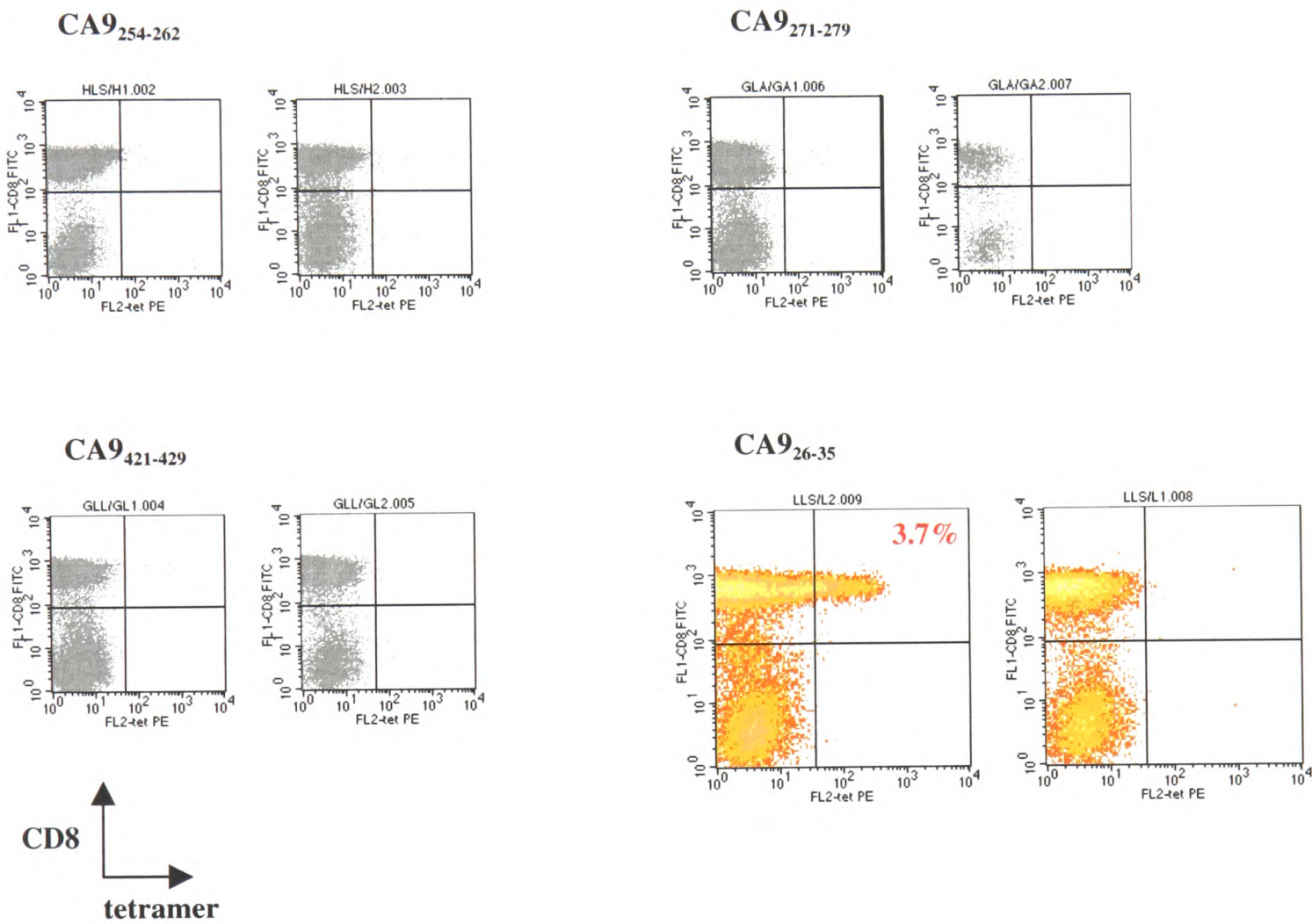
**Fig 5.10.1 In vitro restimulation of CA9<sub>26-35</sub> specific T cells from healthy donor PBL.**  
(A) IFN $\gamma$  ELISpot assay of PBL from 3 healthy donors (H1, H2, H3) following 3 rounds of in vitro restimulation, on day 12 after last restimulation. Cells were pulsed with 10  $\mu$ M peptides derived from CA9 as indicated and control viral peptides, or unpulsed (blank). Each well in duplicate (*red = percentage spots/total number of cells in well*) (B) FACS plots of PBL from corresponding wells on ELISpot plate above, stained with anti-CD8 and HLA-A2/CA9<sub>26-35</sub> tetramer (*red = percentage CD8+ tetramer+ / total number of cells*)



A



B

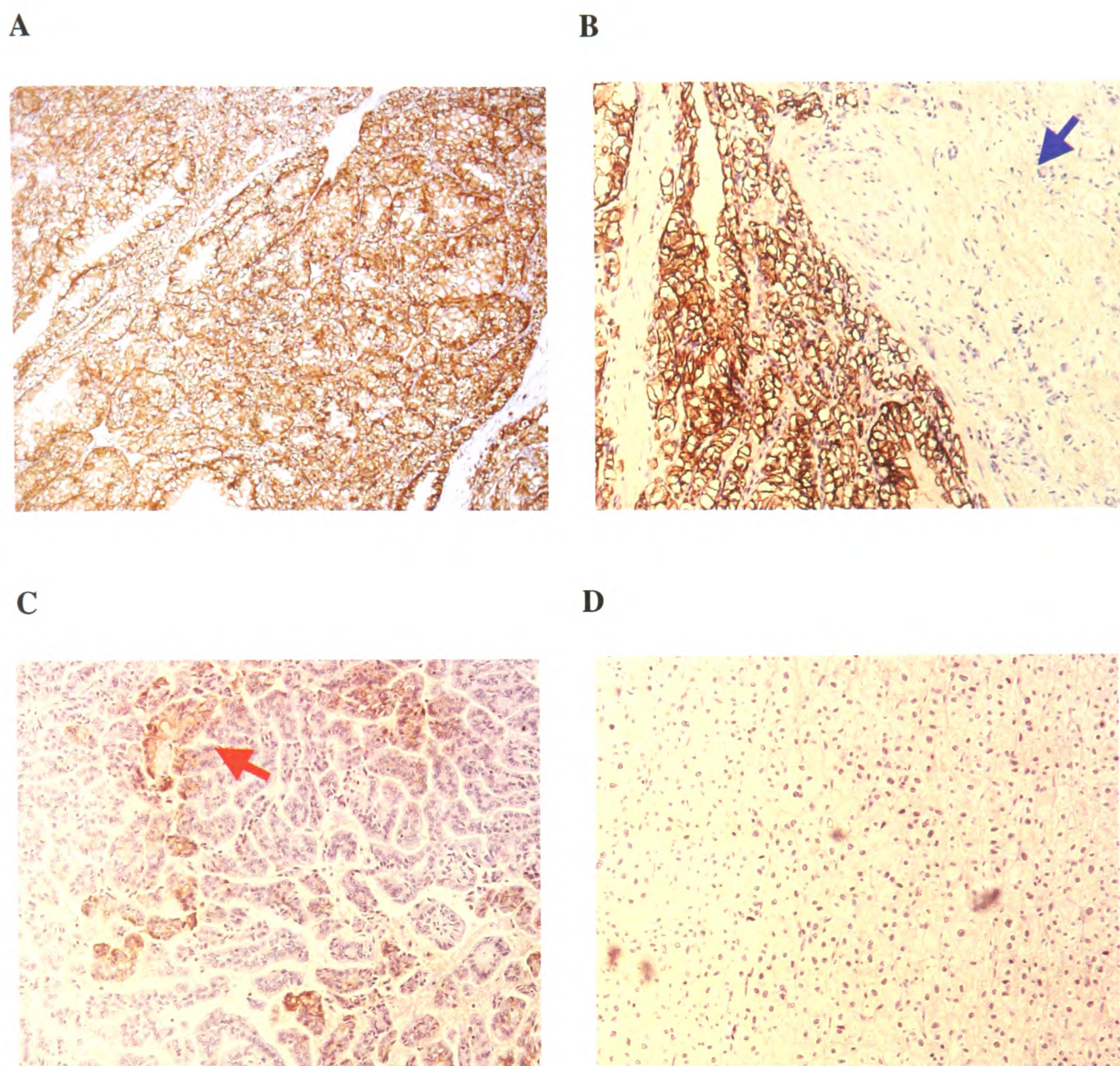


**Fig 5.10.2 In vitro restimulation of CA9 specific T cells from an RCC patient (BYW) PBL.** (A) IFN $\gamma$  ELISpot assay of PBL from an RCC patient following 3 rounds of in vitro restimulation, on day 12 after last restimulation. Cells were pulsed with 10  $\mu$ M peptides derived from CA9 as indicated and control viral peptides, unpulsed (blank), or non-specifically blasted with PHA. Each well in duplicate (*red = percentage spots/total number of cells in well*) (B) FACS plots of PBL from corresponding peptide pulsed wells on ELISpot plate above, stained with anti-CD8 and respective HLA-A2/CA9 tetramer (*red = percentage CD8<sup>+</sup> tetramer<sup>+</sup> / total number of cells*)

| No. | age | sex | histology      | Stage         | Grade<br>(furhman) | CA9 staining           | CA9 peptides |     |     |    | Control peptides |   |
|-----|-----|-----|----------------|---------------|--------------------|------------------------|--------------|-----|-----|----|------------------|---|
|     |     |     |                |               |                    |                        | 254          | 421 | 271 | 26 |                  |   |
| p1  | 50  | f   | clear cell     | confined      | 3                  | -                      | 0            | 0   | 0   | 0  | 0                | 0 |
| p2  | 75  | f   | clear cell     | confined      | 1                  | 3+ membranous/ diffuse | -            | -   | -   | -  | +                | + |
| p3  | 77  | m   | clear cell     | confined      | 2                  | 3+ membranous/ diffuse | -            | -   | -   | +  | +                | + |
| p4  | 80  | f   | clear cell     | confined      | 2                  | 3+ membranous/ diffuse | -            | -   | -   | -  | +                | + |
| p5  | 56  | m   | clear cell     | confined      | 1                  | 3+ membranous/ diffuse | -            | -   | -   | -  | +                | + |
| p6  | 66  | f   | clear cell     | confined      | 2                  | 3+ membranous/ diffuse | -            | -   | -   | -  | +                | + |
| p7  | 37  | f   | clear cell     | confined      | 1                  | 0                      | -            | -   | -   | +  | +                | + |
| p8  | 28  | f   | clear cell     | confined      | 2                  | 0                      | -            | -   | -   | -  | +                | + |
| p9  | 70  | m   | clear cell     | confined      | 2                  | 0                      | -            | -   | -   | -  | +                | + |
| p10 | 68  | m   | clear cell     | local adv     | 3                  | 3+ membranous/ diffuse | 0            | 0   | 0   | 0  | 0                | 0 |
| p11 | 51  | m   | clear cell     | local adv     | 4                  | 3+ membranous/ diffuse | 0            | 0   | 0   | 0  | 0                | 0 |
| p12 | 66  | f   | clear cell     | local adv     | 2                  | 3+ membranous/ diffuse | -            | -   | -   | +  | +                | + |
| p13 | 52  | m   | clear cell     | local adv     | 3                  | 3+ membranous/ diffuse | -            | -   | +   | -  | +                | + |
| p14 | 60  | m   | clear cell     | local adv     | 2                  | 3+ membranous/ diffuse | +            | -   | -   | -  | +                | + |
| p15 | 77  | m   | clear cell     | local adv     | 2                  | 0                      | -            | -   | -   | -  | +                | + |
| p16 | 58  | m   | clear cell     | local adv     | 4                  | 0                      | -            | -   | -   | +  | +                | + |
| p17 | 52  | m   | clear cell     | metastatic    | 3                  | 3+ membranous/ diffuse | -            | -   | -   | -  | +                | + |
| p18 | 50  | m   | clear cell     | metastatic    | 3                  | 3+ membranous/ diffuse | -            | +   | -   | -  | +                | + |
| p19 | 75  | m   | clear cell     | metastatic    | 3                  | 3+ membranous/ diffuse | -            | -   | -   | +  | +                | + |
| m1  | 70  | m   | o              | local adv/met | 0                  | 0                      | -            | +   | +   | -  | +                | + |
| m2  | 78  | m   | o              | local adv/met | 0                  | 0                      | -            | -   | -   | -  | +                | + |
| m3  | 72  | m   | o              | local adv/met | 0                  | 0                      | -            | -   | -   | -  | +                | + |
| m4  | 61  | m   | o              | local adv/met | 0                  | 0                      | -            | -   | -   | -  | +                | + |
| m5  | 44  | m   | o              | local adv/met | 0                  | 0                      | -            | -   | -   | -  | +                | + |
| c1  | 83  | m   | papillary      | o             | 0                  | 3+ membranous/ focal   |              |     |     |    |                  |   |
| c2  | 43  | m   | angiomyolipoma | o             | 0                  | -                      |              |     |     |    |                  |   |
| c3  | 70  | m   | oncocytoma     | o             | 0                  | -                      |              |     |     |    |                  |   |
| c4  | 55  | m   | oncocytoma     | o             | 0                  | -                      |              |     |     |    |                  |   |
| c5  | 58  | m   | papillary      | o             | 0                  | 3+ membranous/ focal   |              |     |     |    |                  |   |

Table 5.10 Analysis of CA9 expression in tumours and HLA-A2/CA9 peptide responses in PBL from RCC patients and controls  
 + : positive   - : negative   o: not done/applicable





**Fig 5.10.3 CA9 immunohistochemistry on RCC tumour sections.** (A) Clear cell RCC at 10x magnification showing global staining of CA9 (brown). Note that tumour cells are organised into tubular structures. (B) Clear cell RCC at 20x magnification showing staining of CA9 in tumour cells with none in adjacent connective tissue (blue arrow). (C) Papillary RCC at 20x magnification showing focal expression of CA9 (red arrow). (D) Chromophobe type RCC at 20x magnification showing no expression of CA9

associated with VHL mutations on chromosome 3. In comparison, other non-clear cell type TCC as the chromophobe variant (fig 5.10.3D) and benign tumours of the kidney (angiomyolipoma, oncocytoma) were also analysed (Table 5.10, c1-5). The table also summarises the IFN $\gamma$  ELISpot data for the four A2 restricted CA9 as well as control viral peptides. There were detectable responses to all four peptides, with a bias towards the CA9<sub>26-35</sub> peptide. Interestingly, there was a suggestion of a correlation of CA9 peptide responses with locally advanced/ metastatic stages compared to localised disease, which did not reach statistical significance (54% vs 33% respectively;  $\chi^2$  test,  $p < 0.2$ ). All patients responded to control viral peptides.

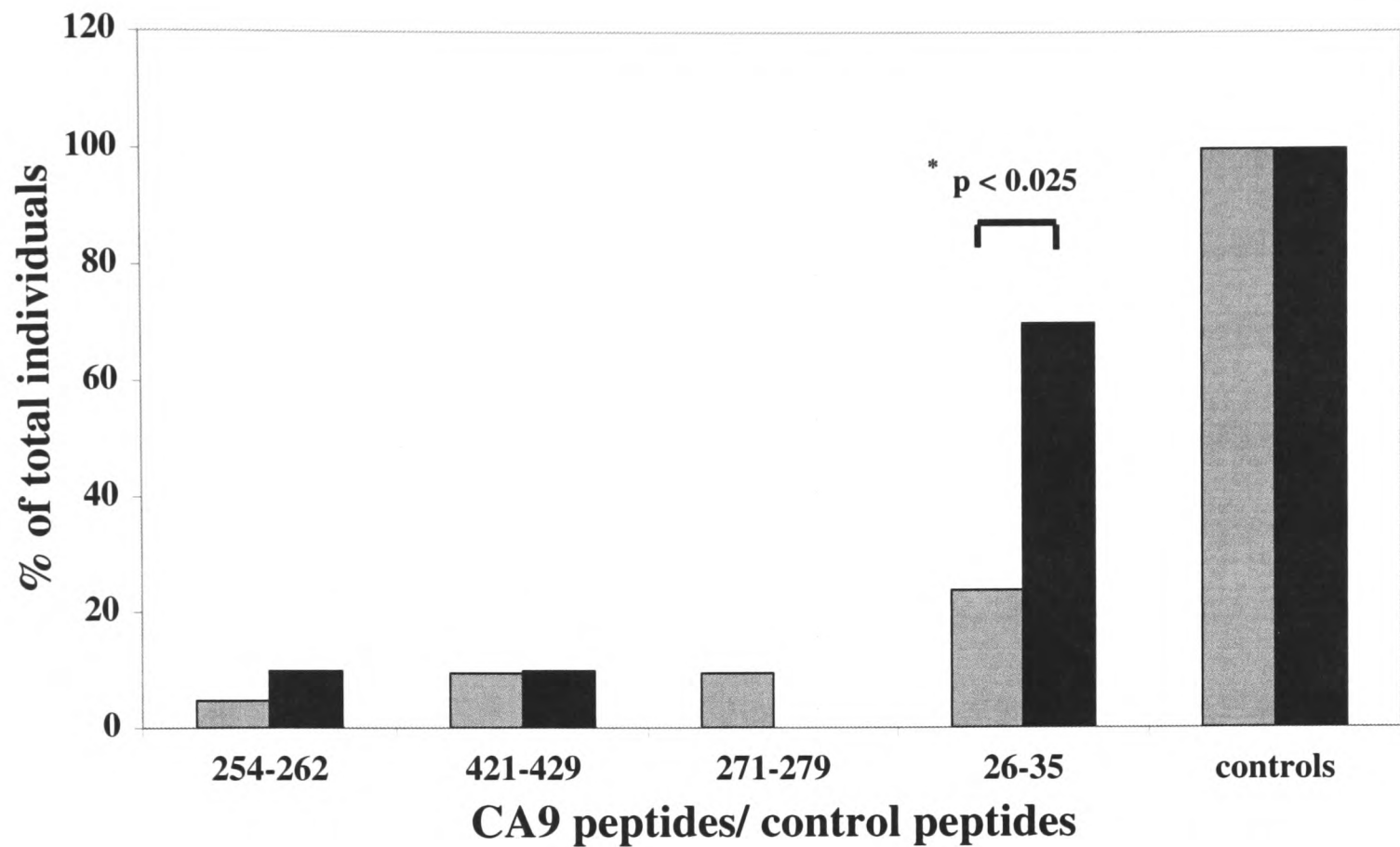
#### **5.11 Natural T cell responses to CA9<sub>26-35</sub> peptide is significantly less frequent in a cohort of RCC patients versus healthy controls**

From the same data set above, the IFN $\gamma$  ELISpot responses to the four A2/CA9 derived peptides between a cohort of RCC patients (n= 24) and healthy controls (n=10) were broadly compared. Interestingly, Fig 5.11 shows that responses to CA9<sub>26-35</sub> peptide was significantly less frequent in RCC patients than healthy controls ( $\chi^2$  test,  $p < 0.025$ ). Differences within the other 3 peptide responses were not statistically significant. All RCC patients and healthy controls demonstrated reactivity to control viral peptides

#### **5.12 A CA9<sub>26-35</sub> specific CTL line derived from a RCC patient (BYW) recognises exogenous but not endogenously presented CA9<sub>26-35</sub> peptide**

The previous data from a CA9 specific CTL line from a healthy donor (A2/CA9<sub>254-262</sub>) suggested a low avidity phenotype (fig 5.5B). Binding affinity data from Vissers *et al*

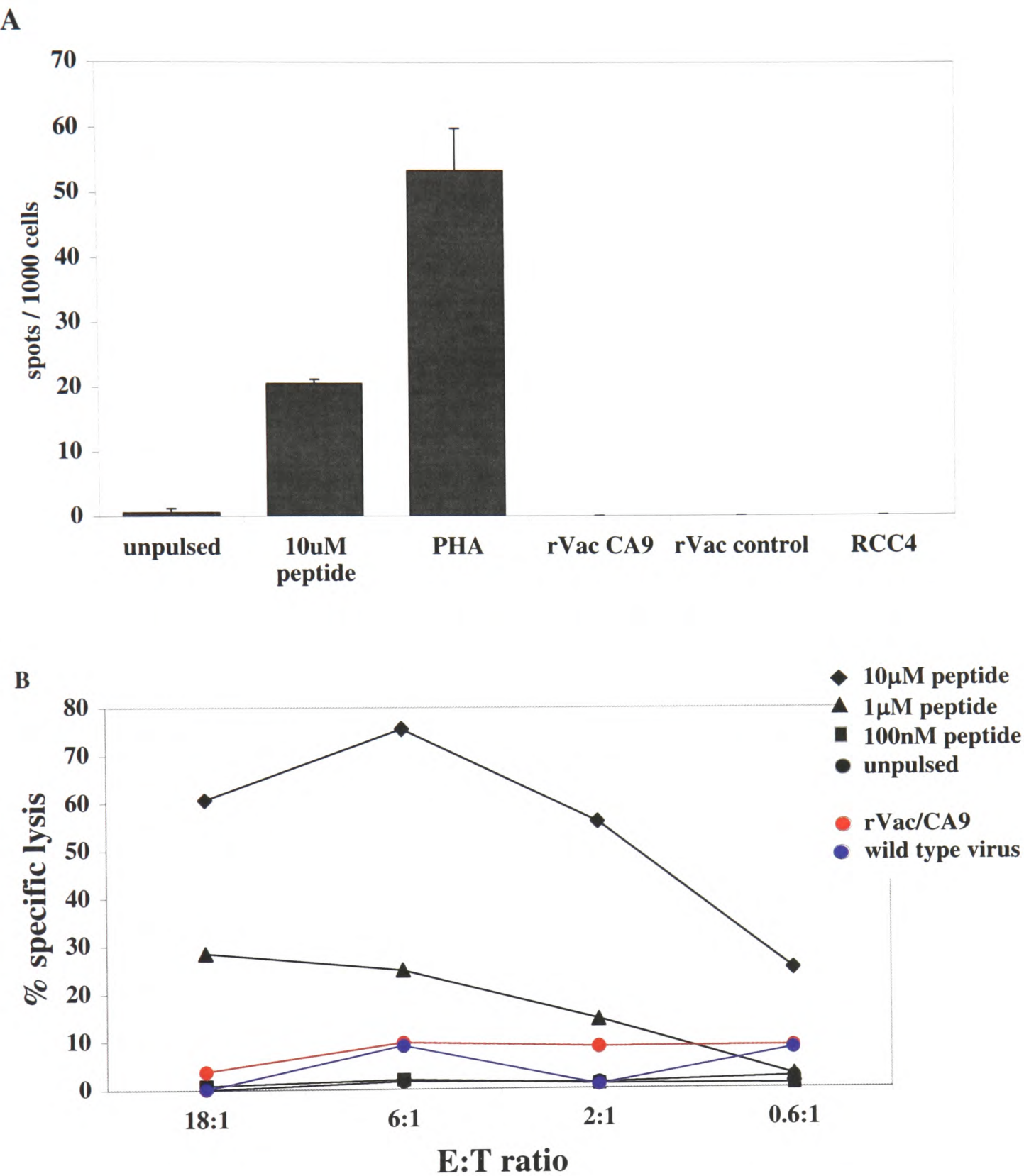




**Fig 5.11 HLA-A2/CA9 peptide responses in RCC patients and healthy controls.** Comparison of ELIspot positive responses in RCC patients (grey bars, n=24) vs healthy controls (black bars, n=10). PBMC from RCC patients and healthy donors were re-stimulated in vitro over 3 rounds with respective CA9 and control peptides. All specimens were analysed in IFN $\gamma$  ELIspot assay 12 days following last re-stimulation. Results depicted as percentage of total individuals with positive ELIspot for respective peptide. (\*  $\chi^2 = 6.09$ ,  $p < 0.025$ )

(Vissers et al., 1999) demonstrated lower affinity of the CA9<sub>254-262</sub> peptide for HLA-A2, than the CA9<sub>26-35</sub> peptide. Therefore it was hypothesised that this higher affinity could translate into higher avidity interactions with A2/CA9<sub>26-35</sub> peptide bearing targets. Using a CTL line derived from a RCC patient BYW (see fig 5.10.2), further enrichment of CA9<sub>26-35</sub> peptide specificity was achieved through A2/CA9<sub>26-35</sub> tetramer staining and FACS. Sorted lines were non-specifically expanded in vitro with allogeneic feeders and PHA, until CA9<sub>26-35</sub> specific CTL reached 40% of sorted cells (data not shown).

These effectors cells were used in an IFN $\gamma$  ELISpot assay against JY targets that were infected with rVac/CA9 or control virus, and a RCC line (RCC4) that constitutively expressed CA9. Both targets were checked for CA9 expression as before. Fig 5.12A shows that neither RCC4 nor rVac/CA9 infected JY targets were recognised, unlike peptide pulsed and control PHA blasted targets. To corroborate the IFN $\gamma$  ELISpot data, a <sup>51</sup>Cr release cytotoxicity assay was performed using the same effector and JY target cells. Fig 5.12B similarly demonstrates that only exogenously loaded peptide resulted in specific lysis by CA9<sub>26-35</sub> CTL. Interestingly, the avidity of this CTL for peptide pulsed targets was improved over the previous CTL line (CA9<sub>254-262</sub>) analysed, with 25% specific lysis detected at 1 $\mu$ M peptide concentration (E:T ratio 6:1) as compared with 5% at a similar peptide concentration (E:T ratio 10:1) in fig 5.5B.



**Fig 5.12 CA9<sub>26-35</sub> specific CTL line from RCC patient BYW recognises exogenous but not endogenously presented CA9<sub>26-35</sub> peptide.** (A) ELISPOT assay using CA9<sub>26-35</sub> specific effectors incubated with JY targets (E:T ratio = 1:100) pulsed with 10μM CA9<sub>26-35</sub> peptide or infected with rVacCA9 or control virus, and CA9 expressing RCC line RCC4. Unpulsed JY cells and PHA blasted JY cells as controls. (*mean of duplicate wells with 1 SD error bars*) (B) <sup>51</sup>Cr release assay to demonstrate specific lysis of JY target cells pulsed with peptide (black) at 10μM (diamond), 1μM (triangle), 100nM (square) and unpulsed (circle); or infected with rVacCA9 (red) and control virus (blue). (*E:T = effector : target* )

### 5.13 Discussion

Notwithstanding that CA9 is a self antigen, it has been demonstrated that CA9 reactive T cell precursors exist in healthy donors and RCC patients. Initially, a CA9 specific line for CA9<sub>254-262</sub> peptide from a healthy donor was generated using an in vitro priming technique. The specificity of the line was confirmed using a novel A2/CA9<sub>254-262</sub> tetramer, and further enriched via a tetramer-based FACS technique. Using this enriched line, functionality in terms of IFN $\gamma$  secretion (fig 5.4) and cytotoxicity (fig 5.5A) when engaged by cognate peptide/MHC was demonstrated. The avidity of the line was low (fig 5.5B), with specific lysis of peptide pulsed targets occurring above 1 $\mu$ M concentration. Not surprisingly, the low avidity of this self-antigen CA9 reactive line has possibly resulted from central tolerance mechanisms during thymic selection (Lee et al., 1999a; Liu et al., 1995; Sebzda et al., 1994), where high avidity CTL have been deleted.

An alternative explanation of the low avidity could have been related to the amount of peptide used to re-stimulate CTL in vitro. Current laboratory practice in the group advocates the routine use of 10 $\mu$ M peptide to pulse APC to generate peptide specific CTL lines *ex vivo* from PBMC. Since natural tumour specific T cell immunity is generally uncommon and of a small precursor frequency (with the exception of tumour antigen Melan-A), the use of high concentrations of peptide in vitro is warranted to maximise the proliferation of tumour specific responses. Nevertheless, Alexander-Miller *et al* (Alexander-Miller et al., 1996b) have demonstrated in vitro that CD8 effector CTL are susceptible to proliferative inhibition by high doses peptide antigen. By performing re-stimulation with low amounts of peptide, they were able to induce CTL that were 100-



fold more sensitive in killing virally infected targets (Alexander-Miller et al., 1996a). The use of 10 $\mu$ M peptide concentration for restimulation in these experiments could have selected for lower avidity CTL in vitro. The low avidity of the CTL line was partially compensated for by induced expression of CA9<sub>254-262</sub> peptide/MHC in JY cells through rVac/CA9<sub>254-262</sub> minigene infection (fig 5.6), resulting in specific lysis of targets. Although Vissers (Vissers et al., 1999) has suggested that the peptide CA9<sub>254-262</sub> was naturally processed and presented in a human melanoma line, the possibility of failure of endogenous processing and presentation in this case remains, since rVac/CA9<sub>254-262</sub> minigene infected targets were lysed, but not recombinant vaccinia encoding full length CA9. Similarly, the RCC4 tumour cell line expressing CA9 was not recognised (fig 5.8). The possibility of a general antigen processing defect in both JY and RCC4 cells was excluded, since these cells when transduced by the rVac/ NY-ESO, were specifically lysed by NY-ESO<sub>154-165</sub> CTL (fig 5.9).

Notwithstanding, the utility of low avidity self-specific CTL in effecting anti-tumour immunity has been demonstrated (Cordaro et al., 2002). Interestingly, such CTL were able to reject tumour while avoiding autoimmunity (Morgan et al., 1998; Speiser et al., 1997). The possibility remains that low avidity CA9<sub>254-262</sub> CTL could prove useful in rejecting CA9 expressing tumours, either through in vitro enhancement of CTL avidity or deliberate induction of CA9 expression through biological response modifiers like type 1 and 2 interferons (Brouwers et al., 2003). Interferons have also been implicated in the up regulation of MHC I and II molecules (Angus et al., 1993; Gastl et al., 1996), as well as

proteins directly involved in the endogenous processing and presentation of peptide in RCC (Seliger et al., 1997; Seliger et al., 1996).

With regard to the influence of membrane bound CA9 on the tumour microenvironment, Newell *et al* have studied glycolysis-deficient cells and concluded that production of lactic acid was not the only factor accounting for peri-tumoral acidity (Newell et al., 1993), which is an intrinsic feature of the malignant phenotype. Griffiths *et al* (Griffiths et al., 2001) have suggested a role for membranous carbonic anhydrases as another contributing cause. Acidic microenvironments have been shown to inhibit anti-tumoral cytotoxicity (Fischer et al., 2000), whilst the stability of peptide/MHC interaction is known to be pH dependent (hence the use of acid elution techniques to analyse cell surface MHC-bound peptides). Inhibition of specific TCR recognition and cytotoxicity in CA9 transduced cells was hypothesised, but was not demonstrated (fig 5.7). The successful recognition and lysis of NY-ESO transduced RCC cells which constitutively express membranous CA9, by NY-ESO<sub>154-165</sub> specific CTL suggests that CA9 was not significantly inhibiting cytotoxicity. The extracellular acidification by membranous CA9 in this case may have not been sufficient to affect peptide/ MHC affinity or CTL avidity. In addition, the abundance of surface peptide/MHC expression in exogenously loaded or virally transduced targets or may have compensated for any peptide/MHC instability induced by the acidic environment. It remains however, a possibility that peri-tumoral acidification by constitutive surface CA9 expression could be sufficient to interfere with naturally processed and presented antigens which are at comparatively lower surface densities than exogenous peptide loading or viral transduction.

The presence of CA9 specific T cells capable of expansion in vitro within a cohort of healthy donors and RCC patients (Table 5.10) was detected. Some investigators have reported in vaccinated patients, that in vitro restimulation of lymphocytes to increase antigen specific T cells results in substantial functional changes from the actual *in vivo* state (Monsurro et al., 2002). In contrast, others have suggested that short term in vitro expansion (< 2 weeks) is not of sufficient duration to generate specific T cells *de novo* from naïve precursors (Nagorsen et al., 2003), and therefore may represent true *ex vivo* responses. The data presented here suggest that natural CA9 antigen experienced T cells are not to be found in either healthy donors or RCC patients, since *ex vivo* IFN $\gamma$  ELIspot analysis as well as short term (<3 weeks) re-stimulation failed to generate CA9 specific responses. The three week duration of in vitro expansion in these experiments before responses were detected probably imply an in vitro priming effect. However, it remains a possibility that CA9 specific antigen-experienced T cells still exist, but were below the detection limits of the assays.

The correlation of IFN $\gamma$  ELIspot secretion and binding of A2/CA9 tetramer in one healthy donor PBMC (H1 in fig 5.10.1) is in line with the reported increased sensitivity of detection by MHC I tetramers over IFN $\gamma$  expression on ELIspot assay within a CD8 population (Klenerman et al., 2002). On the other hand, the discrepancy in another donor (H3 in fig 5.10.1) could be attributed to differential kinetics of interaction of TCR with peptide/MHC multimeric ligands within a polyclonal population (Rubio-Godoy et al., 2001). These same cells were able to produce IFN $\gamma$  in response to cognate peptide,

indicating that efficient signalling had occurred. There are 2 models of TCR activation; the occupancy model posits that the potency of a TCR ligand basically follows the law of mass action, and hence is determined by ligand concentration and TCR-ligand affinity (Sykulev et al., 1995). Hence, the presence of excess peptide in the IFN $\gamma$  ELISpot assay (10 $\mu$ M) resulted in efficient TCR activation. In contrast, the kinetic model suggests that the potency of TCR ligation is governed by the off-rate of TCR-ligand interaction (Rabinowitz et al., 1996). Thus different off-rates with respect to TCR-tetramer interactions within polyclonal populations could result in diminished tetramer staining.

Interestingly, a positive correlation between CA9 peptide responses with tumour stage (Table 5.10) was suggested but which did not reach statistical significance. Others have reported significant correlations between immune responses and tumour stage. For example, Nagorsen *et al* (Nagorsen et al., 2000) reported that natural T cell responses against tumour associated antigens (TAA) occurred more frequently in colorectal cancer patients with lymph node or distant metastases, but not with organ confined disease. This has also been described in melanoma patients with late, metastatic disease who displayed an activated antigen specific CTL phenotype, as opposed to those with early disease (Dunbar et al., 2000). Advanced disease may be required for priming CTL, but is not in itself sufficient since only just over half of the RCC patients with advanced disease (local and distant) developed responses. Of course, the study was limited to four HLA-A2 restricted CA9 epitopes; the presence of other immunogenic epitopes could not be excluded.

A CA9<sub>26-35</sub> specific CTL line from one RCC patient (fig 5.10.2) was isolated and subsequently determined to be of higher avidity compared with a previous CTL line (compare fig 5.12B with fig 5.5B), since specific lysis of peptide pulsed targets on <sup>51</sup>Cr release assay occurred at lower peptide concentrations. This was in line with the peptide/MHC binding data reported by Vissers *et al* (Vissers et al., 1999) which showed superior peptide/MHC binding affinity of the CA9<sub>25-36</sub> peptide over CA9<sub>254-262</sub> peptide. However, despite increased avidity, the CA9<sub>26-35</sub> specific CTL were also unable to recognise endogenously presented peptide on JY or RCC4 targets (fig 5.12). The data suggest that either the avidity of this CTL line was still insufficient to activate TCR, or that the cognate peptide was not processed and presented on the target cell surface. It was not possible to ascertain the reasons for this inability of recognition at this stage of experiments. The use of specific antibodies to peptide/MHC complexes could prove useful in this respect to determine the absence or presence of cognate peptide on the cell surface (Denkberg et al., 2002).

The affinity of peptide/MHC interactions correlate with peptide immunogenicity (Sette et al., 1994). Therefore it was not unexpected that CA9<sub>26-35</sub> peptide elicited the most responses in healthy donor PBMC (fig 5.11). The pre-dominance of this peptide response was not due to competition between CA9 derived peptides, since PBMC were individually re-stimulated in vitro with peptide, except for the control viral peptides which were pooled. The loss of this hierarchy of responses in RCC patients compared to healthy donors was intriguing. The absence of IFN $\gamma$  ELISpot responses from those wells pulsed with the CA9<sub>26-35</sub> peptide in RCC patients correlated with the absence of

A2/CA9<sub>26-35</sub> tetramer staining on FACS analysis (data not shown), indicating that the responding T cells had indeed been lost or deleted rather than rendered unresponsive or anergic. The presence of IFN $\gamma$  reactivity to the control peptides in all patients implies that there was no global T cell anergy to all antigens.

The specific loss of A2/CA9<sub>26-35</sub> reactive CTL could be explained by sequestration of these T cells in to the tumour. Nakano *et al* have suggested that the presence of intra-tumoral CD8<sup>+</sup> T lymphocytes could reflect effective anti-tumour immunity (Nakano et al., 2001). It would have been informative to analyse these TIL for CA9 peptide reactivity via tetramer or IFN $\gamma$  ELISPOT analysis and compare them with PBL from the same patient. Unfortunately, it was not possible to isolate TIL directly *ex vivo* from these RCC specimens with any measure of success. Another explanation could be direct tumour-induced deletion of specific CTL. The apoptosis of TIL and PBL in RCC has been attributed to the ligation of FAS (CD95) on these cells by FAS ligand expressing tumours (Cardi et al., 1998; Uzzo et al., 1999), although recently this phenomenon has been criticised as being spurious (Restifo, 2000). An alternative mechanism of tumour-mediated apoptosis of tumour specific T lymphocytes has come from Dong *et al* who proposed that B7-H1 (a newly characterised member of the B7 co-stimulatory family) expressed on cancer cells could be responsible (Dong et al., 2002). The ligation of PD-1 receptor on activated T cells by B7-H1 was reported to induce apoptosis of tumour reactive T cell clones in vitro. Finally, the resurgence of the putative role of T regulatory cells (T reg) in immune pathology warrants the hypothesis of T reg suppression of CA9 responses. CD4<sup>+</sup>/25<sup>+</sup> T cells have been implicated in the suppression of proliferation and

cytokine responses in CD4<sup>+</sup>/25<sup>-</sup> and CD8<sup>+</sup> T cells both in HIV infection (Kinter et al., 2004) and cancer (Viguier et al., 2004). Depletion of such T reg has been shown to abrogate immunological unresponsiveness and uncover immune responses to shared tumour antigens (Golgher et al., 2002; Shimizu et al., 1999). The tumour antigen specificity of T reg has been elegantly demonstrated by (Wang et al., 2004), while the exact mechanism of suppression remains unclear. Chakraborty (Chakraborty et al., 2004) has reported the importance of IL10 induced suppression by CD4<sup>+</sup>/CD25<sup>+</sup> cells in vaccinated melanoma patients, suggesting a non-specific suppression of CTL responses. The data presented here does not support this, since there were equivalent PBL responses in RCC patients vs healthy donors to control viral peptides (fig 5.11). Therefore, the T reg induced suppression of tumour reactive CTL to the HLA-A2/CA9<sub>26-35</sub> epitope could be a specific phenomenon, resulting in their absence from the peripheral blood.

In summary, the immunogenicity of CA9 in healthy donors and RCC patients has been demonstrated. These T cell responses were of low avidity, and were not able to recognise endogenously presented cognate peptide on target cells unless transduced by the viral minigene. The lack of specific peptide processing/ presentation as a cause of non-recognition remains to be excluded. When in vitro CA9 specific T cell responses in a cohort of healthy donors was compared with RCC patients, the preferential loss of immune responsiveness to peptide CA9<sub>26-35</sub> suggests that this peptide may be presented *in vivo*, thus potentially representing a valuable T cell target for immunotherapy.



## **Chapter 6. Synergistic effect of metronomic dosing of cyclophosphamide combined with specific anti-tumor immunotherapy in a murine melanoma model**

(As published in Cancer Research. vol 63: 8408, 2003, with modifications)

### **6.1 Introduction**

Cytotoxic T lymphocytes (CTL) directed at tumor cells presenting unique peptide on MHC class I molecules constitute a potentially powerful effector arm of host immunity to tumours. Immunization strategies that aim to induce or restimulate tumor-specific CTL may therefore provide an effective and practical approach to cancer treatment (Rosenberg, 2001). While the utility of such immunotherapy has been shown in many preclinical studies, it is unlikely that this treatment modality alone will be sufficient to cure patients with significant metastatic disease, and hence high tumor burdens. In fact, immunotherapy may hold most promise as an adjunct to conventional strategies aimed at cytoreduction, such as chemotherapy (Greenberg, 1991). However, it is well recognized that many chemotherapeutic drugs actively suppress cell-mediated immunity. Cyclophosphamide (CTX), an alkylating agent commonly used in chemotherapy is paradoxical in this regard. While CTX does have immuno-suppressive qualities, and indeed is commonly used as a suppressant in autoimmune conditions such as arthritis and lupus nephritis, it has also been shown to have immune-potentiating activity in some settings (Askenase et al., 1975; Machiels et al., 2001; Maguire and Ettore, 1967;



Mitsuoka et al., 1976; Vierboom et al., 2000). Generally, these potentiating effects have been observed when the drug is administered prior to antigen exposure, or importantly, in low doses (Ehrke et al., 1989; Hengst et al., 1981).

It has recently been reported that proliferating endothelial cells forming new blood vessels within tumours are sensitive to the cytotoxic effects of many anti-neoplastic agents. More significantly, it was recently shown that this anti-angiogenic capacity can be sustained when these agents are administered at frequent intervals, a so-called “metronomic” schedule (Browder et al., 2000; Klement et al., 2000). Metronomic delivery was suggested to be improved over traditional chemotherapeutic regimes involving maximum tolerated doses (MTD) because the latter require extended rest periods to allow the patient to recover from associated toxicity, a respite that also provides time for the endothelial compartment within tumor tissue to repair. In general, the altered drug dosing used in metronomic dosing may be less toxic to normal tissues, and it is therefore possible that some degree of immune function could be maintained under this dosing regime.

In this chapter, it was demonstrated that effective anti-tumor responses could be generated by a synergistic combination of the anti-angiogenic properties of metronomic dosing of CTX, together with specific T cell immunization strategies. The latter was achieved by eliciting CTL reactive to recombinant epitopes expressed by the murine melanoma model B16.F10. The kinetics of immune suppression with metronomic cyclophosphamide dosing was also elucidated.

## 6.2 Sequential immunization of C57BL/6 mice with DNA and vaccinia vectors encoding a tumor-specific antigen can elicit CTL responses capable of resisting tumor challenge.

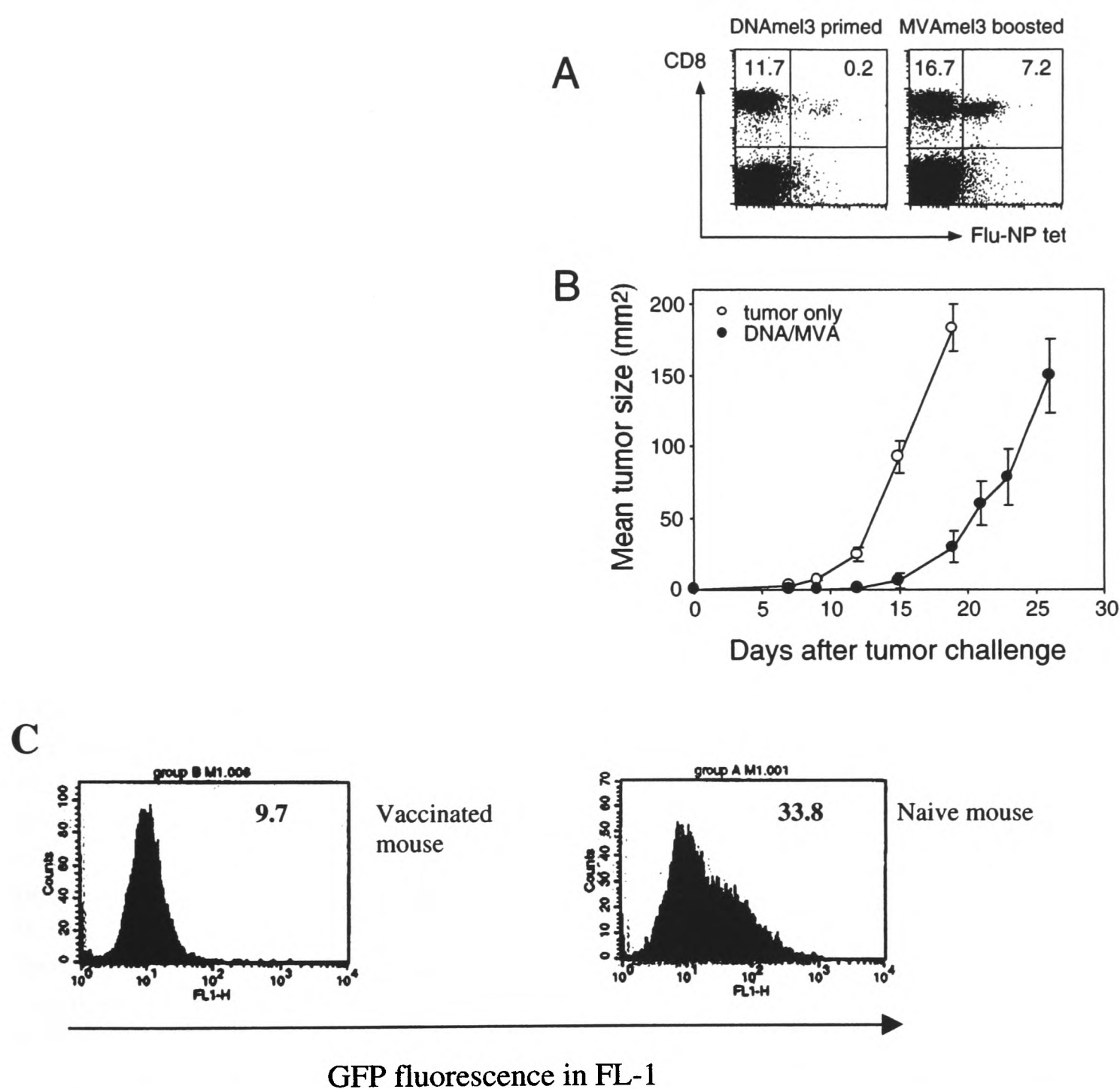
A murine model of melanoma expressing a unique CTL epitope was used to assess specific immune responses to tumours in vivo. It has previously been shown that an immunization strategy involving sequential injection of recombinant DNA and vaccinia vectors encoding a specific epitope can elicit powerful CTL-mediated immune responses (Hanke et al., 1999; Kent et al., 1998; Schneider et al., 1998). Immunization with vectors encoding the polyepitope construct '*mel3*' can be used to initiate CTL responses in both H-2<sup>b</sup> mice and HLA-A2-transgenic mice (Choi et al., 2002). In H-2<sup>b</sup> strains, such as C57BL/6, CTL responses are induced solely to the D<sup>b</sup>-restricted influenza nucleoprotein epitope (NP<sub>366-374</sub>). Sequential immunization with DNA and vaccinia (Modified Vaccinia Ankara strain, MVA) (Carroll et al., 1997) vectors encoding *mel3* was performed to induce NP<sub>366-374</sub>-specific responses capable of resisting challenge with B16 melanoma cells modified to express *mel3* (B16-mel3). DNA (DNA-mel3) was injected intramuscularly to prime NP<sub>366-374</sub>-specific CTL, and then these responses were boosted several days later by i.v. injection of recombinant MVA (MVA-mel3). Proliferation of specific CTL was monitored in the blood using H-2 D<sup>b</sup>/ NP<sub>366-374</sub> tetramers and FACS analysis.

In a typical experiment shown in fig 6.2A, DNA-mel3 alone induced activation and proliferation of NP<sub>366-374</sub>-specific CTL such that by day 13 post-injection the tetramer

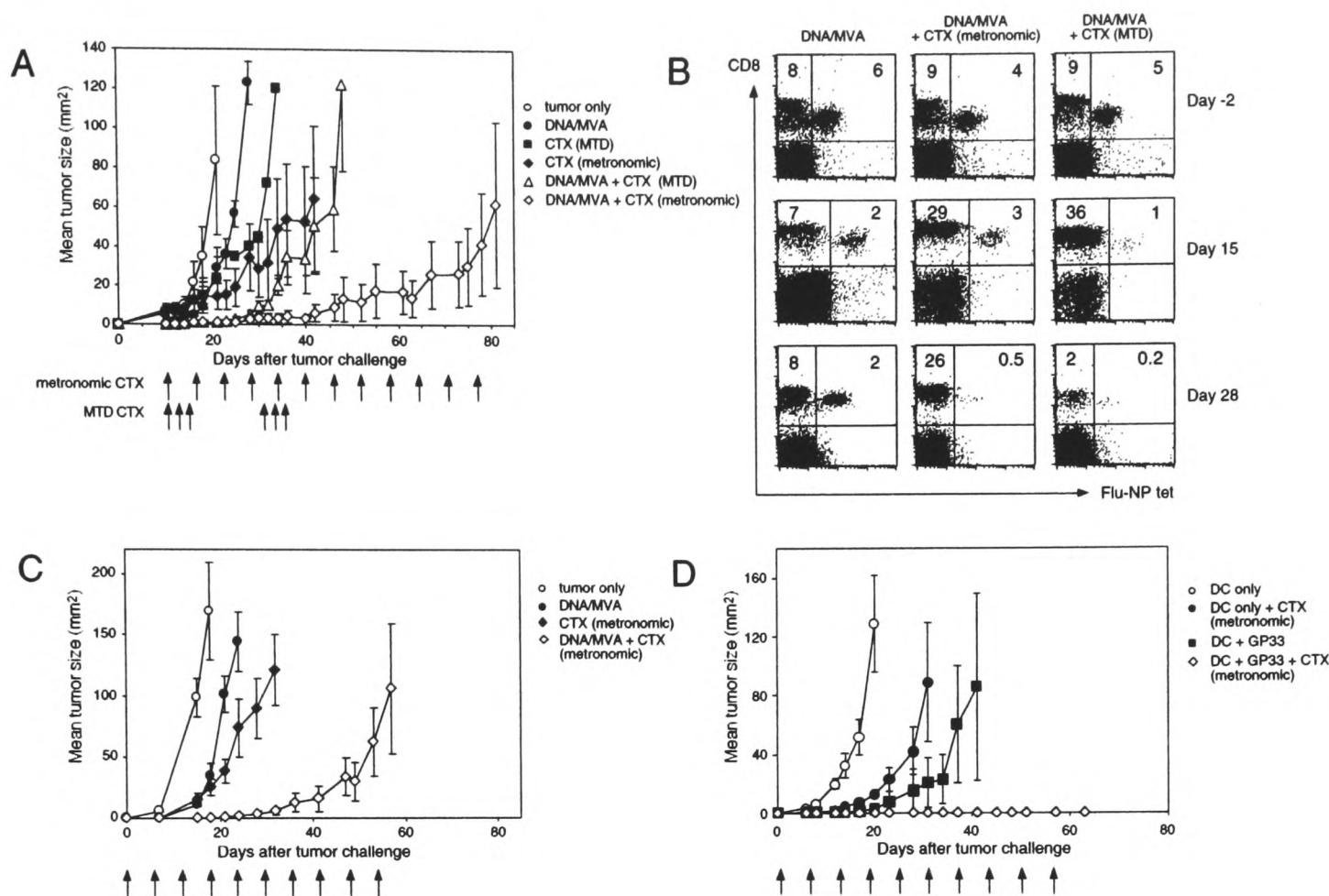
positive population represented an average of  $1.5 \pm 0.21$  % of circulating CD8<sup>+</sup> lymphocytes ( $n = 5$ ). When MVA-mel3 was injected intravenously 14 days after DNA immunization, the NP<sub>366-374</sub>-specific cells were effectively boosted, proliferating to an average of  $25.6 \pm 4.5$  % of CD8<sup>+</sup> lymphocytes 7 days later. For the tumor challenge experiment shown in fig 6.2B, B16-mel3 melanoma cells were injected subcutaneously 7 days after MVA-mel3 boost. These cells had been transfected with the *mel3* polyepitope construct together with a green fluorescent protein (GFP) sequence to monitor antigen expression. Anti-tumour responses were observed in all immunized animals, with engraftment and growth of the administered tumours retarded by 9 days relative to growth in control animals. Analysis of GFP expression in tumours that eventually developed in the immunized animals indicated selective loss of antigen expression (Fig. 6.2C ). These data indicate that this immunization strategy can induce stimulation of significant anti-tumor responses, but immunological escape occurs by antigen loss.

### **6.3 Metronomic dosing with cyclophosphamide enhances antitumour effects of CTL-mediated immunity induced with recombinant vectors.**

The immunotherapy model described above was utilized to investigate the feasibility of combining CTX treatment with anti-tumour immunotherapy (Fig. 6.3A). CTX treatments commenced 10 days post tumour challenge. CTX treatment alone, whether delivered as MTD or metronomic schedule, induced anti-tumour activity resulting in retarded tumor growth relative to untreated controls. Measurements were terminated for each group when the first animal developed a tumor in excess of 200 mm<sup>2</sup>. Thus, while measurements were terminated for the untreated group at day 21 after challenge, animals



**Fig 6.2 Sequential immunization with recombinant vectors encoding a tumor-specific antigen induces resistance to tumor challenge.** CTL responses in C57BL/6 mice were primed by i.m. injection of DNAme13, and then boosted by i.v. injection with MVAmel3 14 days later. **A**, Representative FACS plots showing expansion of a population of Flu-NP<sub>366-374</sub>/D<sup>b</sup> tetramer<sup>+</sup> CD8<sup>+</sup> cells in the blood in response to this immunization regime are presented. Percentages of cells in the upper quadrants are indicated. **B**, Immunized animals were challenged with 3 x 10<sup>5</sup> B16mel3 cells 7 days after MVA boost, and tumor progression was compared with growth observed in unimmunized animals. Mean tumor size per group (n = 5) ± S.E. are shown. **C**, FACS histograms of GFP fluorescence in B16mel3 tumour cells showing loss of antigen expression in tumours in vaccinated vs naïve mice. (figures = mean channel fluorescence)



**Fig 6.3 Combining immunotherapy with metronomic dosing of CTX provides prolonged resistance to tumor challenge.** **A**, Progression of B16-mel3 tumors was monitored in animals that were subjected to one of the following treatment schedules; no treatment (open circles), immunization prior to challenge as outlined in Fig. 1 (closed circles), treatment with CTX administered as MTD (repeated cycles of 150 mg/kg every other day for 6 days followed by 15 day rest) (closed squares), metronomic treatment with CTX (175 mg/kg every 6 days) (closed diamonds), a combination of prior immunization and CTX by MTD (open triangles), a combination of prior immunization and metronomic CTX (open diamonds). Arrows indicate CTX dosing; CTX treatments commenced on day 10 post tumour challenge. Mean tumor size per group ( $n = 5$ )  $\pm$  S.E. are shown. **B**. Blood was taken from immunized animals two days before tumor challenge and 15 and 28 days after challenge in order to examine the effects of CTX treatment on levels of Flu-NP<sub>366-374</sub>-specific CTL, as measured by FACS analysis with Flu-NP<sub>366-374</sub>/D<sup>b</sup> tetramers. Representative FACS plots are shown with percentages of cells in the upper quadrants are indicated. **C**. A repeat of experiment shown in A without groups treated with CTX by MTD, and with metronomic CTX treatment initiated on the day of tumor challenge. **D**. Progression of B16.F10<sub>GP33</sub> tumors was monitored in animals that were subjected to one of the following treatment schedules; immunization with dendritic cells without the tumor-specific antigen GP<sub>33-41</sub> one week prior to challenge (open circles), immunization with dendritic cells loaded with GP<sub>33-41</sub> (closed squares), immunization with dendritic cells without GP<sub>33-41</sub> and metronomic treatment with CTX (175 mg/kg every 6 days) (closed circles), a combination of prior immunization with GP<sub>33-41</sub>-loaded dendritic cells and metronomic CTX (open diamonds).

undergoing the MTD regime survived until day 34 after tumor challenge, and animals receiving the metronomic schedule until day 42 after challenge.

Significantly, the combination of immunotherapy and metronomic dosing of CTX was the most successful of all of the treatment regimes. The first animal in this group bearing a tumor in excess of 200 mm<sup>2</sup> sacrificed at 81 days after challenge (versus 48 days in the immunotherapy/MTD group). Two out of 5 animals were still tumour free at this point, and remained tumour free in excess of 100 days post-challenge in the absence of further CTX treatment, until they too succumbed to tumor. In contrast, all animals in the other groups were bearing tumours at the time measurements were terminated and animals were sacrificed. It is noteworthy that by day 40 post-tumor challenge, when all the CTX treatment groups had received approximately the same overall CTX dose regardless of administration schedule (875 mg/kg for the metronomic group, 900 mg/kg for the MTD group), the combined immunotherapy and metronomic CTX treatment group already had a significantly lower tumor burden than the other groups (mean tumor size for metronomic CTX and immunotherapy =  $3 \pm 2$  mm<sup>2</sup>, versus  $34 \pm 17$  mm<sup>2</sup> for MTD CTX and immunotherapy). These data suggest that a synergistic anti-tumor response when metronomic delivery of CTX was combined with immunotherapy.

#### **6.4 Effects of CTX on lymphocyte numbers in blood.**

Analysis of white blood cell counts from animals involved in the above experiment at day 40 post tumour challenge indicated that all of the groups treated with CTX were leucopenic, although there were no significant differences in cell counts between the drug

treatment regimes (average for all CTX-treated groups =  $1.5 \times 10^3$  cells/mm<sup>3</sup> blood, versus  $10.3 \times 10^3$  cells/mm<sup>3</sup> blood for control animals). However, *ex vivo* FACS analysis of PBL at different time-points over the experiment indicated that there were differences in the kinetic profiles of lymphocytopenia induced by the different drug administration regimes (Fig 6.3B and Table 6.4). Both strategies resulted initially in a reduction of CD8<sup>+</sup> cells in preference to CD8<sup>+</sup> cells as indicated by a higher proportion of CD8<sup>+</sup> cells at day 15 after challenge. Significantly, the metronomic CTX treatment resulted in retention of a higher proportion of NP<sub>366-374</sub>-specific lymphocytes in the blood ( $2.8 \pm 0.4$  %) than in animals receiving CTX at MTD ( $0.9 \pm 0.1$  %), although for both treatment groups the proportion of specific CTL as a percentage of CD8<sup>+</sup> cells was considerably reduced relative to the immunized animals not receiving CTX.

Thus, while the CTX treatment selectively reduced the numbers of tumour-specific CTL, presumably by targeting proliferating cells, this effect was lower in animals undergoing metronomic treatment. This observation was most likely due to the lower overall dose of CTX that these animals had received by day 15 (a total of 175 mg/kg for the metronomic group versus 450 mg/kg for the MTD group). Therefore a significantly larger, potentially tumour-reactive CTL pool was retained in the blood of animals receiving metronomic treatment in the first few days after treatment was commenced. By day 28, the number of NP<sub>366-374</sub>-specific CD8<sup>+</sup> in both CTX-treated groups was at the lower level of detection. Notably, the higher doses of CTX resulted in a more profound reduction in all CD8<sup>+</sup> cells by this later period, which was not observed in the metronomic treatment group. These data suggest that by avoiding some of the cytotoxicity associated with the MTD schedule,



Table 6.4. FACS analysis of groups of animals treated with immunotherapy and CTX<sup>a</sup>

|                            | CD8 <sup>+</sup> Flu-NP tet <sup>+</sup> | CD8 <sup>+</sup> Flu-NP tet <sup>-</sup> | CD8 <sup>+</sup> Flu-NP tet <sup>-</sup> |
|----------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| <b>Day -2</b>              |                                          |                                          |                                          |
| DNA/MVA                    | 3.9 ± 1.1                                | 10.7 ± 0.6                               | 85.4 ± 1.5                               |
| DNA/MVA + CTX (metronomic) | 3.1 ± 1.0                                | 9.3 ± 1.2                                | 87.6 ± 0.9                               |
| DNA/MVA + CTX (MTD)        | 3.3 ± 0.7                                | 11.6 ± 1.3                               | 85.1 ± 0.8                               |
| <b>Day 15</b>              |                                          |                                          |                                          |
| DNA/MVA                    | 2.8 ± 0.4                                | 8.4 ± 0.3                                | 88.7 ± 0.7                               |
| DNA/MVA + CTX (metronomic) | 2.8 ± 0.6                                | 27.7 ± 1.6                               | 69.5 ± 1.0                               |
| DNA/MVA + CTX (MTD)        | 0.9 ± 0.1                                | 34.8 ± 1.6                               | 64.2 ± 1.5                               |
| <b>Day 28</b>              |                                          |                                          |                                          |
| DNA/MVA                    | 1.8 ± 0.5                                | 11.1 ± 0.9                               | 87.0 ± 1.0                               |
| DNA/MVA + CTX (metronomic) | 0.7 ± 0.2                                | 25.7 ± 1.8                               | 73.6 ± 1.7                               |
| DNA/MVA + CTX (MTD)        | 0.2 ± 0.1                                | 2.7 ± 0.4                                | 97.1 ± 0.4                               |

<sup>a</sup>Data taken from groups of animals in experiment in Fig.6.3 A,B, showing mean percentage of lymphocytes with the indicated phenotype ± S.E.



the metronomic schedule permits a longer period of CTL activity, and hence provides a more efficacious combination with immunotherapy.

### **6.5 Effects of timing of CTX therapy on antitumor response.**

For the above experiment, CTX treatment (by either dosing regime) was initiated 10 days after tumor challenge, which was 17 days after MVA boost. Interestingly, when metronomic therapy was initiated on the day of tumor challenge (Fig 6.3 C), the combination therapy was not as potent as in the earlier experiment, although the combination therapy was still greatly enhanced over the other treatment groups. In this experiment the metronomic therapy had been initiated at a time when the CTL proliferation was likely to be peaking after MVA boost.

Thus, these results imply that the toxicity associated with CTX treatment, even at the metronomic doses used, still has detrimental activity on proliferating CTL, thereby weakening the anti-tumor responses. Therefore, while the combination of immunotherapy and metronomic dosing of CTX provides a powerful treatment strategy, prudent timing of chemotherapy relative to immunotherapy may be required to provide the most effective response.

### **6.6 Metronomic dosing with cyclophosphamide enhances antitumour effects of CTL-mediated immunity induced with dendritic cells.**

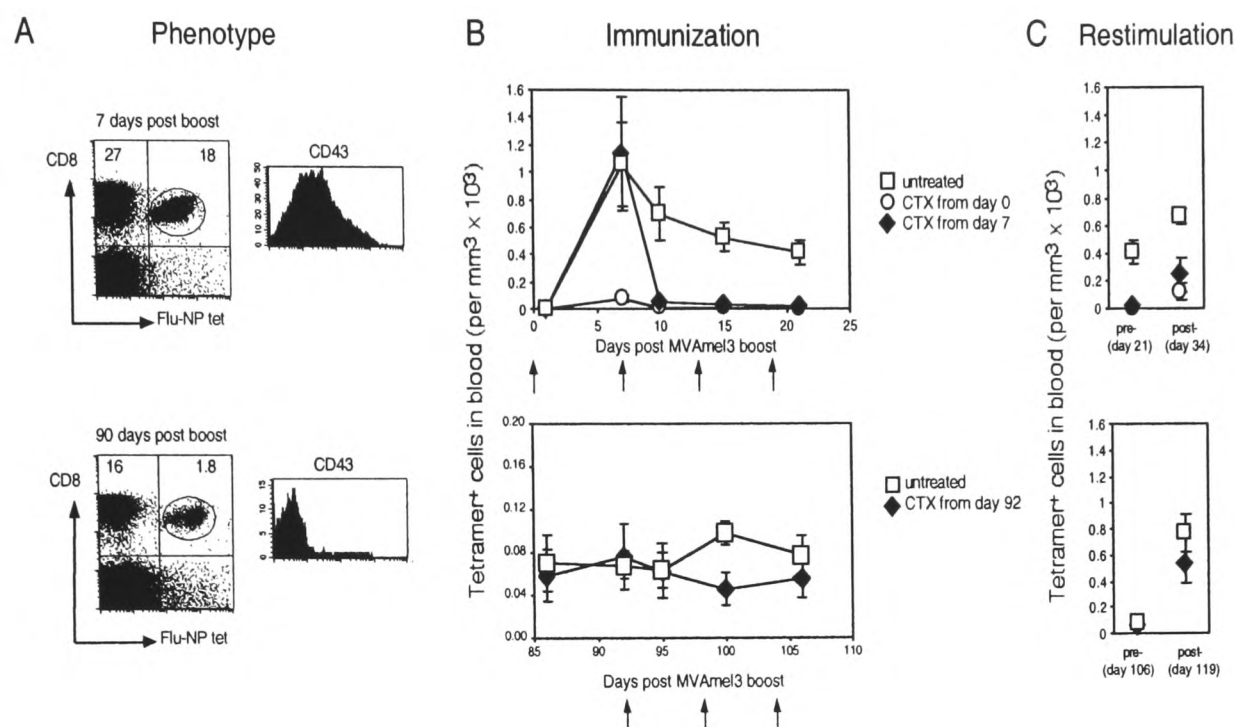
A second B16 melanoma model expressing a minigene encoding the H-2D<sup>b</sup>-binding peptide from LCMV glycoprotein (GP<sub>33-41</sub>) was used to assess the efficacy of metronomic

CTX in combination with immunotherapy administered by injection of GP<sub>33-41</sub> peptide-loaded dendritic cells. One injection of dendritic cells was followed seven days later with challenge of tumor cells and CTX administration. As was observed for the recombinant DNA/MVA strategy above, a synergistic effect was observed with the combined treatment group, with all animals free of tumours when the experiment was ended after 62 days (Fig 6.3D).

### **6.7 Metronomic dosing with cyclophosphamide reduces numbers of proliferating ‘effector’ CTL but spares CTL with re-stimulatory capacity.**

The effect of metronomic delivery of CTX on CTL proliferation and re-stimulatory capacity was studied in the context of immunization with recombinant mel3 vectors as outlined for the experiments above, but without subsequent tumor challenge. Two cohorts of animals were examined; one that had been recently immunized, and another that had been immunised 3 months previously. Phenotypic analysis indicated that CTL in the recently immunized (7 days post boost) expressed high levels of CD43, a phenotype associated with CD8<sup>+</sup> T cell ‘effector phase’ (Fig. 6.4A upper panel) (Harrington et al., 2000). In contrast, analysis of CTL in animals immunized 3 months previously showed low levels of CD43 expression, a phenotype that has been associated with CD8<sup>+</sup> T cell memory (Fig. 6.4A lower panel).

CTX was administered to these animals by metronomic schedule at different times relative to MVA-mel3 boost in order to observe the effect of timing of CTX treatment on numbers of NP<sub>366-374</sub>-specific cells detected in the blood. For these analyses, total



**Fig 6.4 Activated CTL with a CD43<sup>lo</sup> phenotype are resistant to metronomic CTX-mediated deletion, and retain potent restimulatory capacity.** **A.** CTL responses were initiated by immunization as in Fig. 1, and then blood was taken for FACS analysis on the indicated days. Percentages of cells in the upper quadrants are indicated. CD43 expression was assessed on gated CD8<sup>+</sup> Flu-NP<sub>366-374</sub>/D<sup>b</sup> tetramer<sup>+</sup> cells. **B.** CTL responses were monitored in the blood of immunized animals following metronomic CTX treatment initiated on the days indicated. The schedule of CTX administration is indicated with arrows. The total number CD8<sup>+</sup> Flu-NP<sub>366-374</sub>/D<sup>b</sup> tetramer<sup>+</sup> cells per mm<sup>3</sup> of blood was extrapolated from FACS data combined with lymphocyte counts determined using Micro60 blood counter. Data are presented as mean number of CD8<sup>+</sup> Flu-NP<sub>366-374</sub>/D<sup>b</sup> tetramer<sup>+</sup> cells per treatment group (*n* = 5) ± S.E. **C.** Each of the treatment groups in **B** was injected with Vacc-mel3 infected syngeneic splenocytes in order to restimulate Flu-NP<sub>366-374</sub>-specific CTL (day 21, top panel; day 106, lower panel). The mean number of CD8<sup>+</sup> Flu-NP<sub>366-374</sub>/D<sup>b</sup> tetramer<sup>+</sup> cells in the blood per group prior to injection (pre-) and 7 days after injection (post-) are shown.

numbers of NP<sub>366-374</sub>-specific cells in the blood were extrapolated from white blood cells counts, thereby taking into account the general reduction in numbers of white blood cells observed in CTX-treated animals. The effect of CTX on NP<sub>366-374</sub>-specific responses was found to be dependent upon the timing of initiation of drug treatment (Fig. 6.4B top panel). Thus, when CTX treatment was initiated on the same day as MVA boost, CTL proliferation was significantly impaired relative to immunized animals that did not receive CTX ( $p = 0.043$ , Student's  $t$  test), with total numbers of CTL at day 7 post-boost reduced by 90 %. Delaying drug treatment a further 7 days permitted initial CTL proliferation. However, these recently stimulated cells were also susceptible to CTX treatment, and numbers were rapidly reduced after drug treatment was initiated. Within 3 days of CTX treatment, the numbers of specific CTL were reduced by 92 % relative to immunized animals that did not receive CTX. In contrast, when CTX was given to animals that had been primed and boosted 3 months previously, the levels of NP<sub>366-374</sub>-specific CTL in the blood were not affected by drug treatment (Fig. 6.4B lower panel).

Potent *in vivo* re-stimulatory capacity is a hallmark of T cell memory. To further investigate whether metronomic CTX treatment spares cells with a memory phenotype, each of the groups of animals above was subjected to a boosting regime involving intravenous injection with syngeneic splenocytes that had been infected *ex vivo* with the recombinant vaccinia virus Vacc-mel3. This strategy was used because the previous MVA infection would have generated antibodies capable of neutralizing a second vaccinia virus infection. By administering splenocytes that had been infected *ex vivo*, these neutralizing antibodies could be avoided temporarily, permitting presentation of the

NP<sub>366-374</sub> peptide, and hence allowing expansion of NP<sub>366-374</sub> CTL. CTX treatment was terminated 6 days before injection of infected splenocytes. Increases in numbers of NP<sub>366-374</sub>-specific CTL following injection of mel3 vaccinia infected splenocytes were observed in all animals, although this expansion was not statistically significant in animals that had received CTX during or soon after MVA boost (day 0 or day 7 after boost) (Fig. 6.4C upper panel). In contrast, significant increases in NP<sub>366-374</sub>-specific CTL were observed in those animals that were boosted over 90 days previously ( $p = 0.036$ , Student's  $t$  test) (Fig. 6.4C lower panel), with boosting responses similar to control groups that were not treated with CTX. These data demonstrate that CTX treatment causes reduction in the numbers of proliferating CTL, but does spare a cohort of cells with re-stimulatory capacity.

## 6.8 Discussion

Bolus administration of chemotherapeutic drugs at the MTD is known to be associated with considerable toxicity and discomfort to cancer patients. In light of recent studies describing the potent anti-angiogenic capacity of drug administered in a metronomic fashion (Browder et al., 2000; Klement et al., 2000), it is likely that metronomic dose regimes will find favor in the clinical setting. The study presented here highlights another important advantage of metronomic drug treatment, namely that the lowered associated toxicity permits a degree of immune function, and hence the potential to combine drug treatment with immunotherapy. Specifically, it has been shown that metronomic dosing of CTX combines very effectively with anti-tumor immunotherapy administered by injection of recombinant vectors encoding a tumor-specific antigen. These therapeutic combinations could potentially be applied to a variety of neoplasms for which tumour antigens have been defined.

The balance between CTX-induced cytotoxicity on the one hand, and induction of effective immunity on the other, is particularly fine. While metronomomic administration of CTX caused significant reduction in CTL numbers in the blood immediately following initiation of drug treatment, the combination therapy was still dramatically enhanced over either treatment alone. Significantly, the combination of metronomic CTX and prime boost-mediated immunity was more potent than combination therapy using MTD of the drug. This result highlights the importance of reducing CTX-induced cytotoxicity directed at the immune effector cells. Supporting this suggestion, FACS analysis indicated that more CTL persist in the blood of animals receiving the metronomic treatment compared

to animals receiving the MTD. Interestingly, where metronomic dosing was used on immunized animals without tumour challenge, the CTL appear to succumb to the CTX more rapidly (compare fig 6.3B middle column CTX from day 10; and fig 6.4B upper panel CTX from day 7). It is possible therefore that the presence of tumor may provide some CTL restimulation, thereby sustaining numbers in the blood for a longer period. Despite the detrimental effect of CTX on numbers of CTL, the combined antitumor activity of CTL and CTX was significant, implying that sufficient CTL survive to have impact. It should be pointed out that for purposes of tracking immune responses over time, all CTL analyses were performed by monitoring the blood. Homing of lymphocytes in the presence of CTX was not addressed in these studies, and it remains possible therefore that redistribution of CTL could account for some of the observed changes in CTL frequencies in the blood.

It is not yet clear whether the anti-angiogenic environment created by the metronomic scheduling of CTX has any potentiating role with respect to CTL function. The use of alternative anti-angiogenic agents that, unlike CTX, have no direct cytotoxic activity on the tumour cells, remains to be explored. It is likely that the efficacy of the combination therapy is a result of efficient CTL-mediated cytolysis in the first instance, which effectively reduces the tumour burden upon which the drug must act. Thus, while loss of antigen following immunotherapy alone results in unimpeded tumor growth, sustained CTX treatment is able to keep growth of the antigen-loss variants in check for a considerably enhanced period. Alternatively, the CTX treatment, whether by minimizing angiogenesis, or by acting upon the tumor cells directly, may be preferentially removing



proliferating tumor cells. The remaining cells, with a slower proliferative capacity, may take more time to provide antigen-loss variants that can avoid CTL-mediated deletion. Interestingly, the CD8<sup>+</sup> fraction of lymphocytes seemed to be more resistant to the cytotoxic effects of CTX than the CD8<sup>-</sup> cells. This trend was more pronounced under the metronomic regime than the treatment with MTD. It is possible that this CD8<sup>+</sup> fraction contains CTL specific to tumour-specific antigens other than NP<sub>366-374</sub> that contribute to the overall anti-tumor immune response, perhaps through epitope spreading. Additionally, the CD8<sup>-</sup> population prone to CTX mediated cytolysis may include regulatory T cells such as the CD4<sup>+</sup>CD25<sup>+</sup> population shown to suppress anti-tumor activity in other studies (Onizuka et al., 1999; Shimizu et al., 1999), although such regulatory T cells are not known to proliferate. Indeed, removal of a suppressor population has been proposed as one of the mechanisms providing the previously reported immune potentiating effects of doses of CTX prior to immunotherapy (Glaser, 1979). Yet another possible explanation for the synergy between metronomic cyclophosphamide and specific anti-tumour immunotherapy could be drug-mediated increases in tumour immunogenicity (Giampietri et al., 1981; Tentori et al., 1995), or cyclophosphamide-induced alterations within the tumour stroma (Ibe et al., 2001).

The doses of CTX used in this study were taken directly from the report of Browder et al (Browder et al., 2000), in which the anti-angiogenic property of metronomic low dose CTX therapy was first reported. These experiments were also performed on C57BL/6 mice, with the metronomic dose provided as approximately one third of the MTD. It should be noted that some weight loss and discomfort was observed in animals that were



subjected to metronomic dosing for periods in excess of 90 days. Thus it may still be desirable to lower the dose even further. A recent report has shown that CTX can be administered constantly in drinking water, effectively lowering CTX dose while sustaining antiangiogenic capacity (Man et al., 2002). It is likely that such a strategy would result in less immune suppression, and less associated morbidity.

The effect of CTX on numbers of CTL in the blood appears to be dependent on the phenotype of these antigen-experienced cells. While even the metronomic schedule induces a rapid reduction in numbers of recently activated cells, this reduction is avoided if drug administration is delayed a period of months after MVA boost, by which time the majority of activated lymphocytes had reverted to a CD43 low phenotype. This phenotype has been described by Harrington *et al.* (Harrington et al., 2000) to be a memory phenotype. Our experiments show that these cells retain a significant re-stimulatory capacity, a hallmark of memory, regardless of whether they had been exposed to CTX or not (Fig. 3C). CTX is a well-recognized alkylating agent, with its activity directed at the DNA of dividing cells. It is not surprising therefore that CTL in a proliferating “effector” phase fall prone to the activity of this drug, while cells activated months earlier, and approaching quiescence, do not.

It should be possible to exploit this knowledge in the design of combination treatment schedules. Where primary treatment either through surgical extirpation or radiation therapy has not been curative, or where the risks of developing subsequent metastatic disease are high, combination treatment could be administered in an adjuvant setting.

Immunotherapy could be initiated well before metronomic dose CTX administration to minimize immunosuppression. With the first signs of recurrent disease, the tumour-specific memory compartment can be effectively boosted by immunotherapy during a temporary respite from metronomic dosing. Alternatively, in the setting of advanced local or metastatic disease, MTD chemotherapy could be initiated at sufficiently high doses to debulk the tumour, followed by combination therapy as described.

## **Chapter 7. A mycobacterial iron chelator desferri-exochelin induces hypoxia inducible factor, NIP3 and VEGF in cancer cell lines**

(As published in Can Res. vol 62: 6924, 2002, with modifications)

### **7.1 Introduction**

Mammalian cells adapt to a hypoxic environment by increased expression of specific hypoxia- inducible genes. A key player in this repertoire of responses is hypoxia-inducible factor (HIF), whose levels are stabilized by hypoxia (see Introduction). The oxygen sensing mechanism is also sensitive to iron chelation. It has been shown that the iron chelator deferoxamine mesylate (DFX) is capable of inducing hypoxia regulated gene expression (Wang and Semenza, 1993; Wenger, 2000). A key mechanism for oxygen sensing has recently been shown by two groups (Ivan et al., 2001; Jaakkola et al., 2001) to be a proline hydroxylase, which is an iron containing tetramer. Iron chelation inhibits activity of this enzyme (Ivan et al., 2001; Jaakkola et al., 2001) and prevents the post translational modification of HIF that targets it for degradation by the VHL complex.

Exochelins are iron chelators secreted by pathogenic strains of *Mycobacterium tuberculosis*. They are both lipid and water soluble, rapidly enter cells, have a high binding affinity for ferric iron, and prevent iron-mediated redox reactions (Gobin and Horwitz, 1996; Gobin et al., 1995; Gobin et al., 1999; Horwitz et al., 1998). The exochelin used in our study was the synthetic form of desferri-exochelin 772 SM (Keystone Biomedical Inc, Los Angeles CA, USA) which is chemically and functionally

indistinguishable from the most prevalent native desferri-exochelin derived from *M tuberculosis* (Pahl et al., 2000). This agent has been shown to induce cell death by apoptosis in MCF-7 and T47D-YB breast cancer cell lines and was considerably more potent than deferoxamine in this regard (Pahl et al., 2001).

As alluded to in the Introduction to this thesis, the augmentation of the hypoxic response in tumours could potentially be useful in combination with hypoxia-directed T cell immunotherapy. The results in this chapter demonstrated that in MDA468 breast cancer cells, desferri-exochelin 772SM induced HIF by iron chelation and activated downstream hypoxia responsive genes, including the expression of the angiogenic protein, vascular endothelial growth factor (VEGF), and the pro-apoptotic protein NIP3 (Bruick, 2000). The potency of this highly diffusible, lipophilic iron chelator was compared to the poorly diffusible, lipid-insoluble iron chelator deferoxamine. Iron being an essential co-factor in many cellular processes, it was anticipated that iron chelation could lead to other consequences apart from HIF induction, and the effects on cell proliferation independent of HIF were studied. Since desferri-exochelin has been reported to cause growth arrest in vascular smooth muscle cells (Pahl et al., 2000), the anti-angiogenic effects of desferri-exochelin in vitro was also investigated.

## **7.2 Desferri-exochelin stabilizes HIF in MDA468 breast cancer cells**

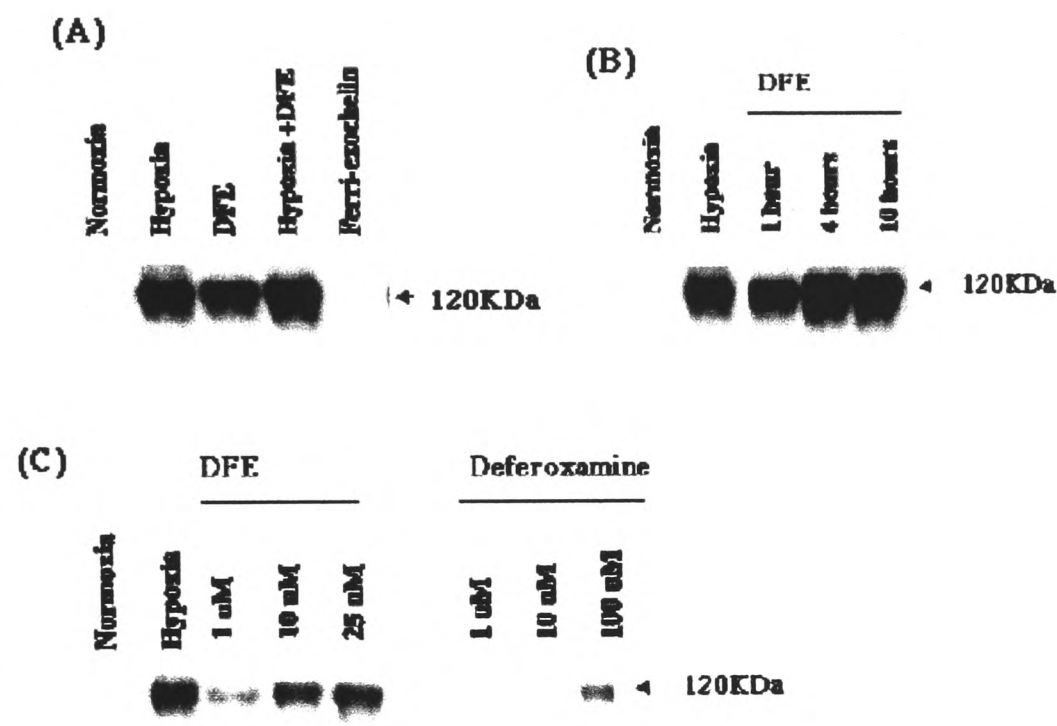
Sixteen hour exposures to either hypoxia 0.1% or desferri-exochelin 772SM caused stabilization of HIF-1 $\alpha$  protein detected by Western blots in MDA 468 breast cancer cells (Figure 7.2A). Concomitant exposure to both hypoxia and desferri-exochelin did not

reveal any significant additive effects on HIF-1 $\alpha$  expression. Exposure to iron-loaded ferri-exochelin did not result in stabilization of HIF-1 $\alpha$ . Stabilization of HIF-1 $\alpha$  by desferri-exochelin occurred by 1 hour, reached maximal levels by 4 hours, and did not increase further between 4 and 10 hours exposure (Figure 7.2B). Desferri-exochelin stabilized HIF-1 $\alpha$  at doses 1 log less than were required for deferoxamine for the same effect (Figure 7.2C).

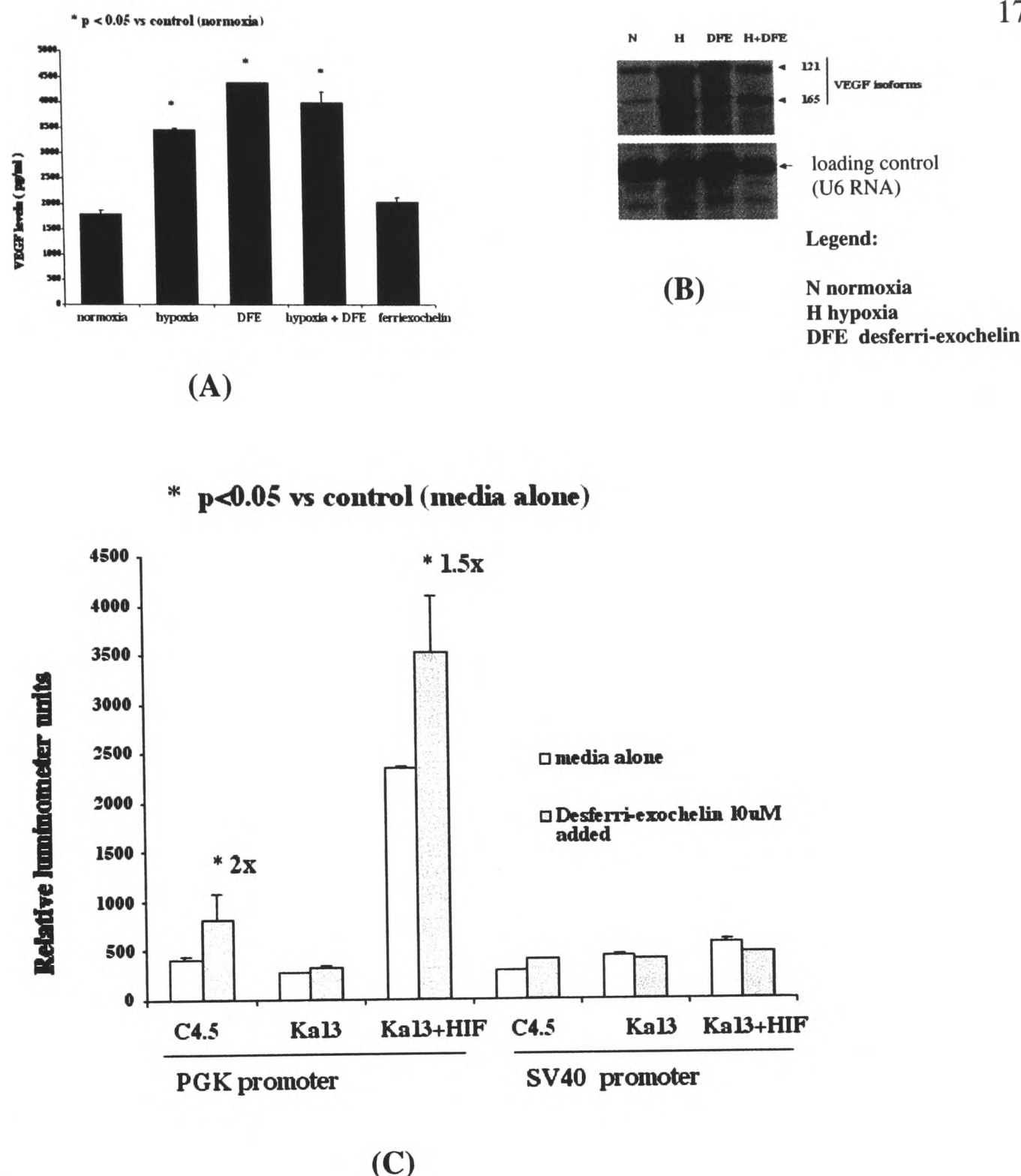
### **7.3 Desferri-exochelin induces VEGF in MDA468 breast cancer cells**

VEGF is the most well studied hypoxia-inducible protein, and is the principal factor behind hypoxia-induced angiogenesis. VEGF levels were measured by enzyme-linked immunosorbent assay (ELISA) in culture media from the same samples assayed in Figure 1. Exposure to either hypoxia or desferri-exochelin increased VEGF levels compared with levels during normoxia alone. There were no differences in VEGF levels among exposures to hypoxia, desferri-exochelin, or the combination of hypoxia and desferri-exochelin. Exposure to ferri-exochelin did not increase VEGF levels significantly (Figure 7.3A). Therefore, iron chelation was required for induction of VEGF by desferri-exochelin.

RNase protection assays were performed on MDA 468 cells exposed to 10uM desferri-exochelin 772SM with normoxia as control. After correction for loading variation, there was a 3-6 fold induction of VEGF transcripts with exposure to hypoxia, desferri-



**Fig 7.2 Desferri-exochelin stabilizes HIF-1α in MDA 468 cells.** (A) Western blot for HIF 1α (120 kDa), under conditions of normoxia, 16 hours hypoxia (0.1%O<sub>2</sub>), 16 hours exposure to desferri-exochelin (DFE) 10uM and ferri-exochelin 10uM. (B) Western blot time course experiment for HIF-1α with desferri-exochelin (DFE) 10uM with normoxia and hypoxia controls. (C)Western blot comparing HIF-1α stabilisation by desferri-exochelin (DFE) and deferoxamine at escalating doses.



**Fig 7.3. Desferri-exochelin induces VEGF expression via HIF.** (A) ELISA to measure VEGF expression in media of MDA468 cells in normoxia, 16 hours hypoxia (0.1%  $O_2$ ), 16 hours exposure to desferri-exochelin (DFE)10uM, hypoxia with DFE 10uM and ferri-exochelin 10uM. (Samples in quadruplicate; sample mean with 1 SD shown. Comparison of means (vs normoxia control) using ANOVA and Dunnett's multiple comparison test ( $p < 0.05$ )) (B) RNAse protection assay on MDA468 cells in normoxia (N), 16 hours hypoxia (H) at 0.1%  $O_2$ , and 16 hours exposure to desferri-exochelin (DFE)10uM, and hypoxia with desferri-exochelin (H+DFE). Loading controls were taken from small nuclear RNA (U6 RNA) which were not responsive to hypoxia. (C) Luciferase reporter assay on C4.5, Ka13 and HIF-restored Ka13 cells to demonstrate induction of HRE in the PGK promoter (absent in SV40 promoter). (Samples in duplicate; comparison of means (media alone vs desferri-exochelin treated) using two-tailed independent Student's T test ( $p < 0.05$ ))



exochelin or hypoxia/desferri-exochelin compared to normoxia alone. There were no differences in VEGF RNA expression among the samples from the three exposures (Figure 7.3B). The protection bands appear as a doublet, with the upper band corresponding to the VEGF 121 amino acid isoform, and the lower to the VEGF 165 amino acid isoform. These isoforms arise from alternative exon splicing, and have different biological activities. The VEGF 121 riboprobe protects a 517 nucleotide sequence in the VEGF 121 transcript, but only 439 nucleotides in the VEGF 165 transcript due to interrupted protection from an intervening non-complementary sequence. Hence the protected VEGF 121 transcript migrated slower than the bigger isoform VEGF 165.

The murine hypoxia-regulated enzyme phosphoglycerate kinase (PGK) responds to HIF induction through hypoxia-response elements (HRE) located within the promoter regions of the gene. In Chinese hamster ovary (CHO) C4.5 cells, exposure to desferri-exochelin resulted in induction of a hypoxia-regulated reporter gene (PGK HRE-linked luciferase) (Figure 7.3C). In HIF1- $\alpha$  mutated CHO cells (Ka13) in which HIF expression is absent, no increase in luciferase expression was noted in cells exposed to desferri-exochelin. However transfection of HIF-1 $\alpha$  back into Ka13 cells restored induction of luciferase-HRE PGK by desferri-exochelin. Therefore, HIF-1 $\alpha$  was required for HRE induction by desferri-exochelin.

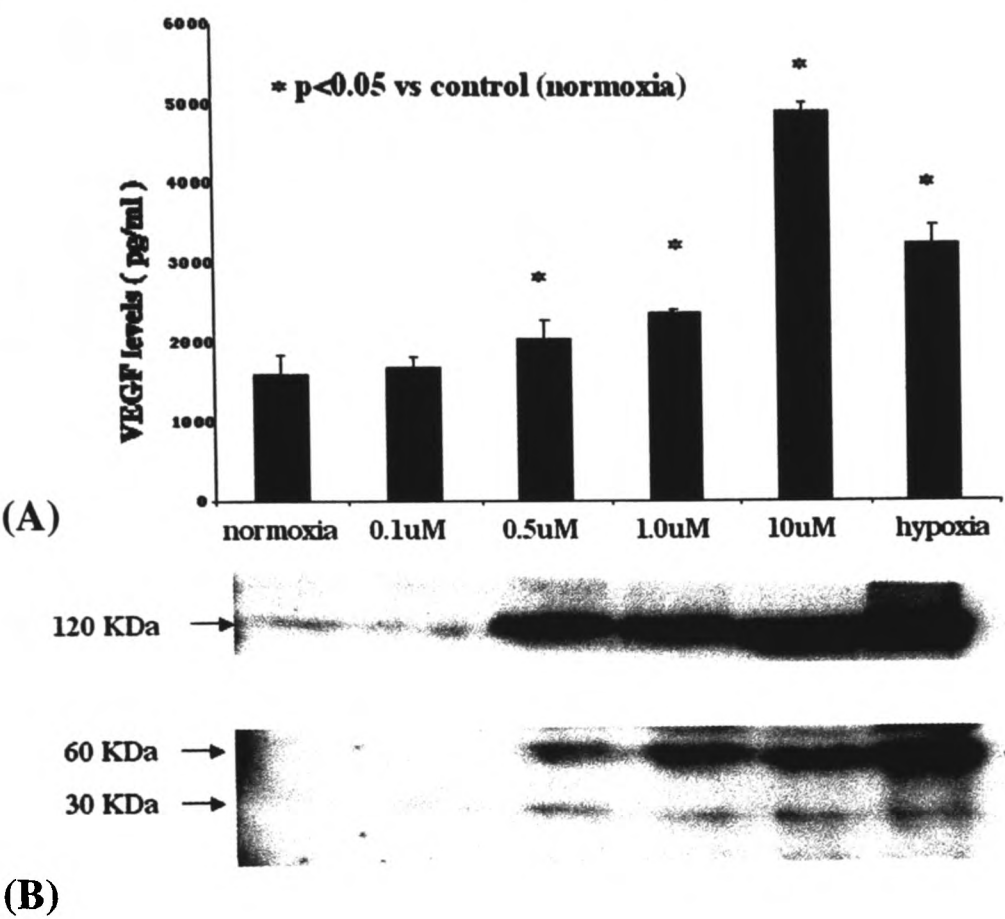
#### **7.4 Desferri-exochelin induces pro apoptotic protein NIP3 in MDA468 breast cancer cells**



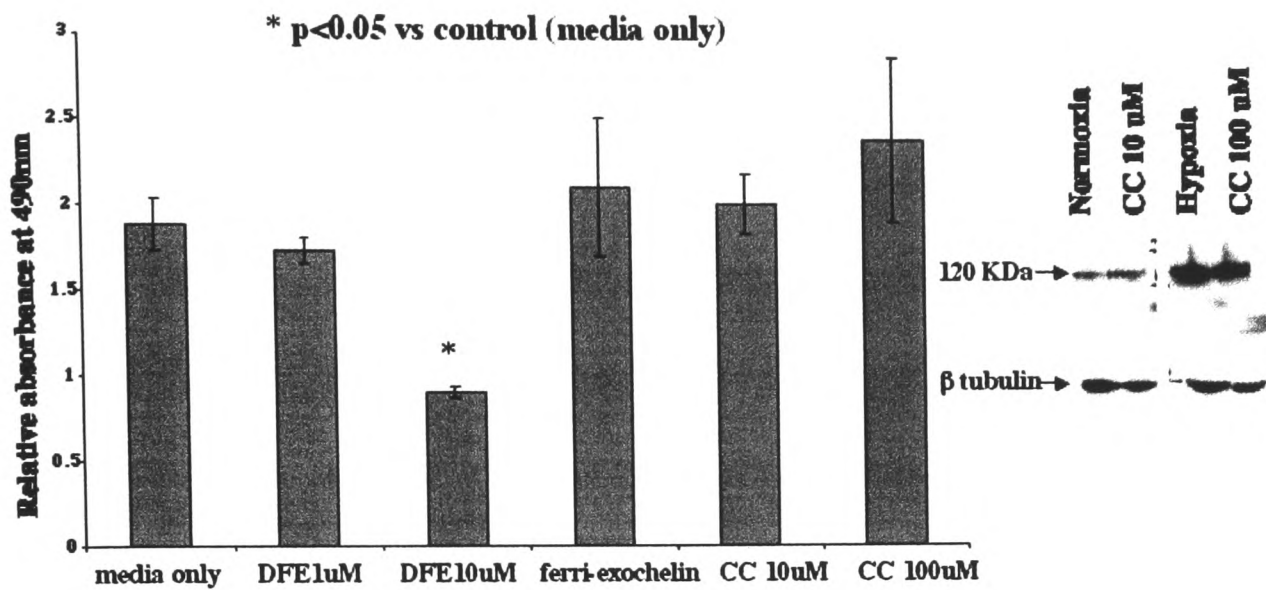
Western blots for NIP3 were performed after MDA 468 cells were treated with various doses of desferri-exochelin over 16 hours, with normoxic and hypoxic controls. Although NIP3 protein is calculated to have a molecular mass of 21 kDa, transiently expressed NIP3 protein has been shown to migrate as both 30 and 60 kDa forms (Sowter et al., 2001). Induction of both forms of NIP3 by desferri-exochelin was detected from 0.5  $\mu$ M concentration (Figure 7.4A). To compare the dual effects of desferri-exochelin on VEGF and NIP3 induction in MDA468 cells, VEGF levels measured by ELISA were compared directly with western blots for NIP3 expression (Figure 7.4B). Both VEGF and NIP3 showed similar induction over normoxic controls from 0.5  $\mu$ M desferri-exochelin, with increasing expression at higher doses (1.0 and 10  $\mu$ M).

### **7.5 Desferri-exochelin inhibits cell proliferation independent of HIF induction**

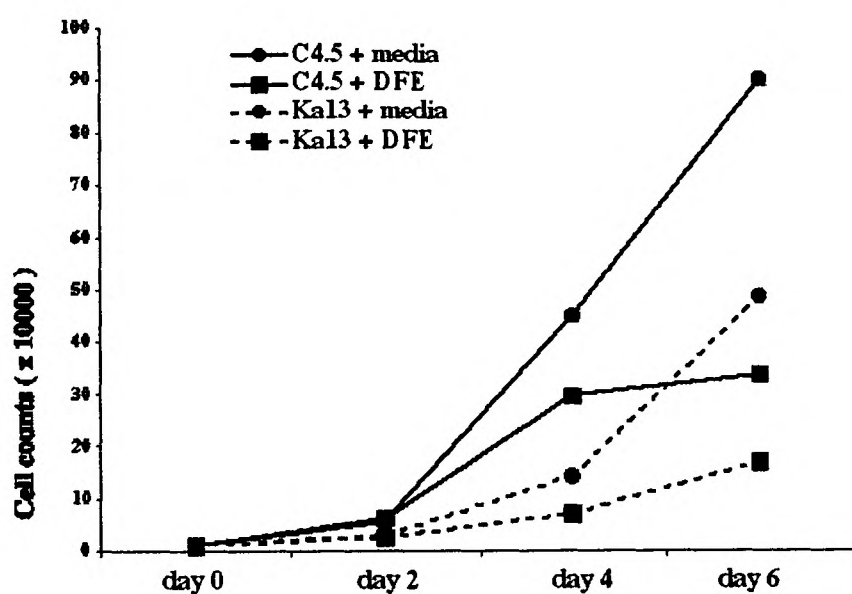
The inhibition of cell proliferation by desferri-exochelin was demonstrated in both malignant and non-malignant cell lines (viz. MDA468 and Chinese hamster ovary CHO cells). Since cobalt chloride is another chemical hypoxia-mimic through stabilisation of HIF that does not involve iron chelation, its effects on MDA468 cell proliferation was compared with desferri-exochelin. Fig 7.5A shows a dose dependent inhibition of MDA468 cell proliferation, with significant inhibition at 10 $\mu$ M concentrations. Iron-loaded ferri-exochelin did not have any effect, indicating that inhibition of cell proliferation by desferri-exochelin was iron chelation specific. In contrast, HIF-inducing doses of cobalt chloride (10 and 100  $\mu$ M) failed to inhibit cell proliferation. Therefore, inhibition of MDA468 cell proliferation by desferri-exochelin was independent of HIF induction.



**Fig 7.4 Desferri-exochelin induces VEGF and NIP3 in a dose dependent manner.** MDA468 cells were maintained in culture in the presence of varying doses of desferri-exochelin (A) ELISA to measure VEGF levels in media after exposure to desferri-exochelin (0.1, 0.5, 1.0, 10uM). Normoxia and hypoxia controls shown. VEGF samples in triplicate. (Sample mean with 1 SD shown. Normoxia controls in both. Comparison of means (vs normoxia control) using ANOVA and Dunnett's multiple comparison test ( $p < 0.05$ ) (B) Western blot for NIP3 (30 and 60 kDa) and HIF (120 kDa) protein with similar escalating doses.



(B)



**Fig 7.5 Desferri-exochelin inhibits cell proliferation in MDA468 and CHO cells independent of HIF.** (A) MTS assay of MDA468 proliferation after 24 hours exposure to drugs desferri-exochelin (DFE) 1 and 10 uM, ferri-exochelin 10 uM and cobalt chloride (CC) 10 and 100 uM. Samples in quadruplicate. (mean with 1 SD shown. Comparison of means (vs media only control) using analysis of variance and Dunnett's multiple comparison test ( $p < 0.05$ ). Western blot insert shows HIF-1 $\alpha$  (120 kDa) induction in hypoxia and cobalt chloride 100 uM. (B) CHO cell variants C4.5 (HIF intact) and Ka13 (HIF mutated) grown in culture. Cell counts at various time points of C4.5 (solid lines) and Ka13 (dashed lines) cells in 6 well plates with or without desferri-exochelin (DFE) 10 uM added to media. Samples in duplicate. (mean with 1 SD shown).

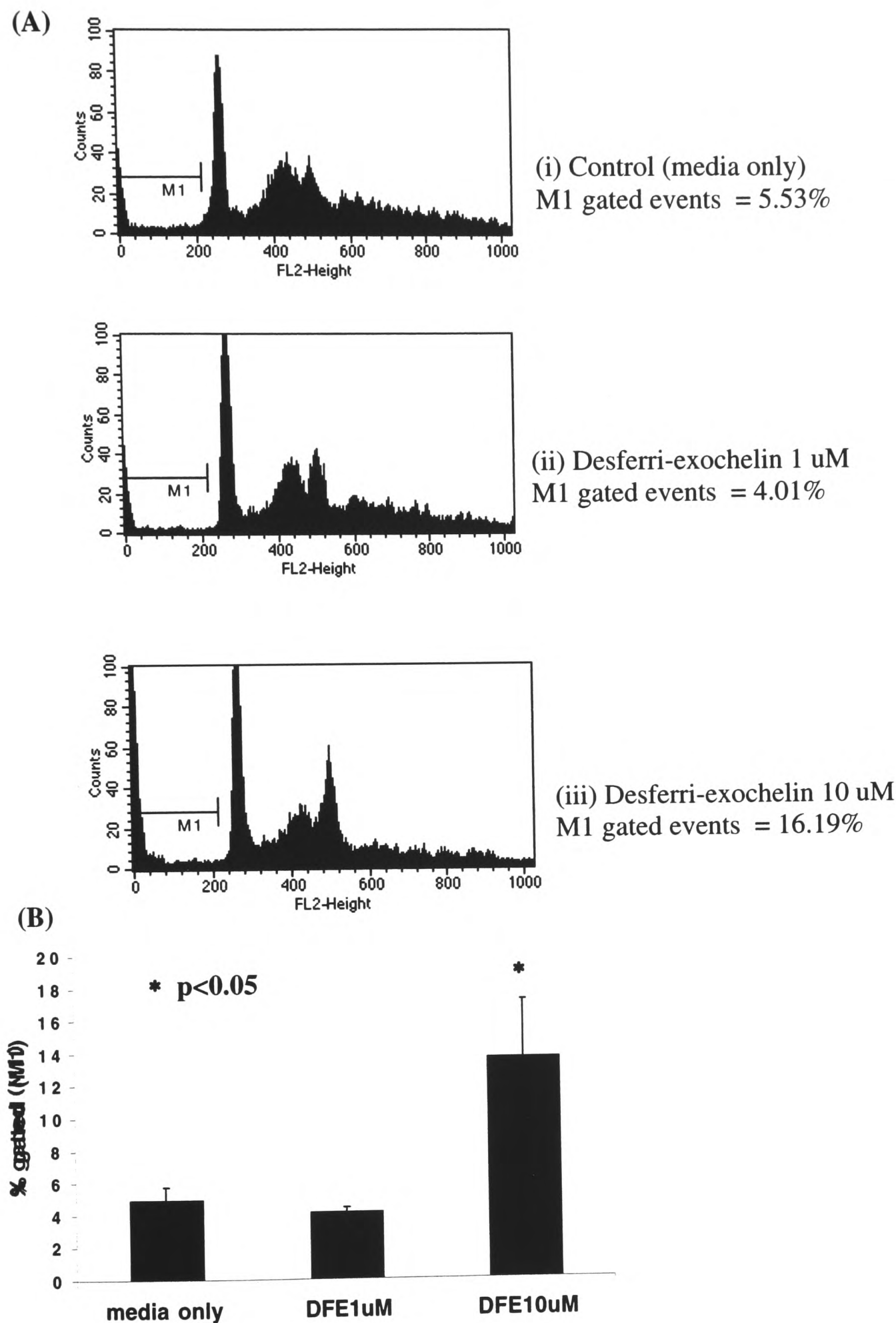
In another system, CHO cells and their derivative Ka13 which lack HIF expression were used as targets for cell proliferation inhibition by desferri-exochelin. Figure 7.5B shows that cell proliferation was slower in Ka13 compared with CHO cells. However, proliferation in both cell types were inhibited by 10 $\mu$ M of desferri-exochelin, indicating that desferri-exochelin inhibition of cell proliferation in CHO cells was independent of HIF.

### **7.6 Desferri-exochelin inhibits MDA468 proliferation through apoptosis induction**

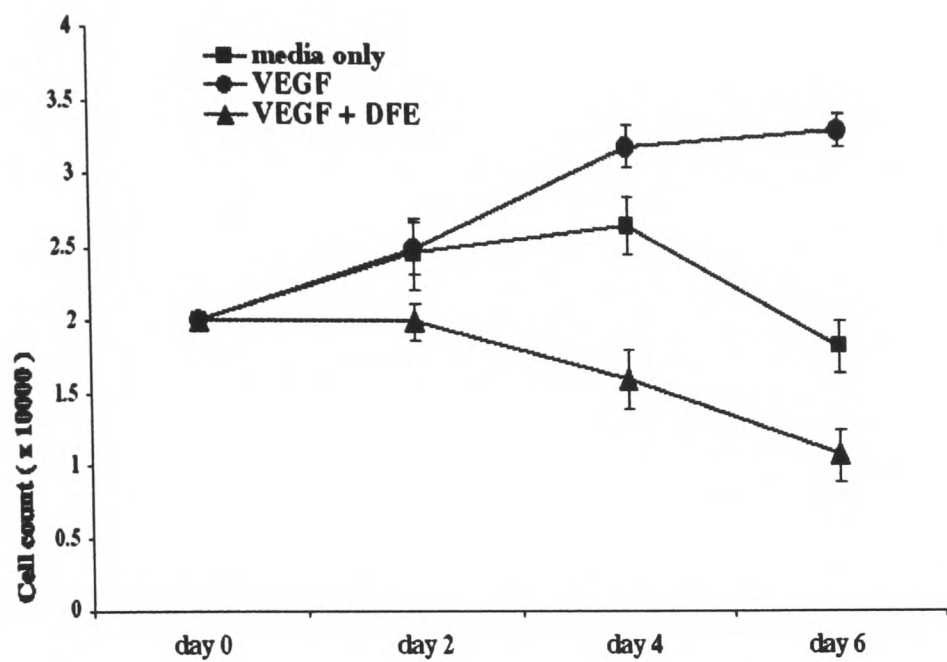
To demonstrate that the inhibition of MDA468 proliferation was due to apoptosis induction and not cell cycle arrest, FACS analysis of desferri-exochelin treated MDA468 cells was performed after staining with DNA marker propidium iodide. Figure 7.6 shows a dose dependent effect on apoptosis induction, with significant apoptosis in MDA468 cells treated with 10 $\mu$ M of desferri-exochelin, consistent with the data from figure 7.5A. Therefore, desferri-exochelin inhibits cell proliferation through induction of apoptosis.

### **7.7 Desferri-exochelin inhibits endothelial cell proliferative response to VEGF**

To assess the effects of desferri-exochelin on endothelial cell growth in the presence of the angiogenic growth factor VEGF, cell proliferation in human dermal microvascular endothelial cells (HuDMEC) in the presence of these factors was measured. The addition of VEGF increased endothelial cell proliferation above that of media alone. Desferri-exochelin however, inhibited this VEGF-induced cell proliferation (Figure 7.7). Therefore, desferri-exochelin displays anti-angiogenic properties in vitro that overcome the pro-angiogenic effects of VEGF.



**Fig 7.6. Exochelin inhibits MDA468 proliferation through increased cell death and not cell cycle arrest.** (A) FACS analysis of permeabilised MDA468 cells stained with propidium iodide and fluorescence detected in FL-2 channel. Figures representative of treatment groups (i) media only , (ii) Desferri-exochelin (DFE) 1 uM, (iii) DFE 10 uM. (B) M1 (apoptosis) gated events from (A) expressed as a percentage of all events. Samples in duplicate. (*mean with 1 SD shown. Comparison of means using analysis of variance and Tukey multiple comparisons test ( $p < 0.05$ )*)



**Fig 7.7 Desferri-exochelin inhibits VEGF induced proliferation of human dermal endothelial cells (HuDMEC).** Cell counts at various time points of HUDMEC grown in 6 well plates with VEGF (8 ng/ml) and desferri-exochelin (DFE)10 uM added to media in some wells. Drugs were pulsed at day 0, 2 and 4. (*Samples in duplicate; mean with 1 SD shown*).

## 7.8 Discussion

It has been demonstrated that desferri-exochelin 772SM can stabilize HIF, and that this effect was dose dependent under normoxic conditions. Desferri-exochelin probably acts at least partly through the same pathway as hypoxia in view of the lack of an additive effect when both were present. The addition of iron to a desferri-exochelin eliminated its ability to induce HIF. Therefore, this action of desferri-exochelin is solely related to iron chelation. Desferri-exochelin stabilized HIF within one hour. It is likely that the lipid solubility of desferri-exochelin allowed it to quickly enter cells and bind iron intracellularly. In support of this, it has also been shown that desferri-exochelin required a smaller dose (1 log less) to achieve a similar effect to the lipid-insoluble iron chelator deferoxamine, which does not readily enter cells. Pahl et al (Pahl et al., 2000) have similarly shown that this desferri-exochelin was ten times more effective than deferoxamine in inhibiting vascular smooth muscle cell proliferation. Horwitz et al (Horwitz et al., 1998) reported that the relative protective effects of different forms of desferri-exochelin in cardiac myocytes exposed to hydrogen peroxide corresponded to their lipid solubility, with the most lipid-soluble forms having the greatest effect. The increased intracellular iron binding ability of desferri-exochelin is believed to be due to its enhanced diffusion across lipophilic cell membranes.

Hypoxia induces a complex program of gene expression involving both pro- and anti-apoptotic pathways (Dong et al., 2001; Graeber et al., 1996; Koong et al., 2000; Maxwell et al., 1999). Pathways including that which enhance angiogenesis and tumour growth (VEGF), and that which promotes tumour cell death by apoptosis (NIP3) were studied.



Both pathways were activated by iron chelation with desferri-exochelin. NIP3 is a mitochondrial protein that is induced by hypoxia (Bruick, 2000; Sowter et al., 2001), and these results have now demonstrated that it is induced by iron chelation as well. Recently, it was shown that hypoxia may regulate a specific necrosis-like pathway via NIP3 (Sowter et al., 2001), through mitochondrial permeability transition pore opening and dysfunction (Vande Velde et al., 2000). Hypoxia selects for survival of more aggressive cancer cell variants that express mutant p53 (Graeber et al., 1996). Since NIP3 does not require p53 for apoptosis, induction of NIP3 by desferri-exochelin may be of therapeutic value. Additionally, iron deprivation may have other effects on cancer cell growth, so HIF induction of NIP3 may not be the sole pathway by which desferri-exochelin kills cancer cells. Pahl et al (Pahl et al., 2001) demonstrated that iron chelation with desferri-exochelin 772SM induces cell death by apoptosis in cultured breast cancer cells, but not in normal breast epithelial cells. Consistent with this, the data have demonstrated iron chelation-dependent inhibition of MDA468 proliferation by desferri-exochelin through increased cell death independent of HIF induction, with no effects on cell cycle.

It was found that by stabilizing HIF with desferri-exochelin, both VEGF and NIP3 were upregulated in MDA468 tumour cells at the same levels of desferri-exochelin (0.5  $\mu$ M). Transcriptional upregulation of VEGF by desferri-exochelin was demonstrated, and this effect has also been demonstrated to be HIF dependant. This precludes a 'therapeutic window' at which pro-apoptotic effects via NIP3 could occur while minimising VEGF induction. Notwithstanding, the presence of VEGF did not prevent the inhibitory effects



of desferri-exochelin on endothelial cell growth. Therefore desferri-exochelin exhibited anti-angiogenic effects even though VEGF induction occurs.

The novel iron chelator exochelin, in its desferri-exochelin form, could potentially enhance the anti-tumour effects of hypoxia-directed T cell immunotherapy. By stabilisation of HIF and induction of HIF-dependent genes, desferri-exochelin could augment the expression of hypoxia-regulated antigens. Its pro-apoptotic effects via NIP3 may be further enhanced by HIF-independent growth inhibitory pathways related to its iron chelating abilities. Furthermore, desferri-exochelin could be anti-angiogenic *in vivo* despite the induction of VEGF, by targeting tumour endothelial cell proliferation. On the other hand, the specific effects of iron chelation on the immune response is not clear, although the importance of iron for proliferation of immune cells is highlighted by the association of iron deficiency states with defects in both humoral and cellular immunity. The presence of the transferrin receptor involved in iron uptake, on immature proliferating thymocytes is another piece of evidence of the importance of iron in T cell development (Bowlus, 2003). Conversely, the association of non-classical MHC HFE complex with transferrin receptor suggests that T cells may in turn regulate iron metabolism (Salter-Cid et al., 2000).

## Chapter 8. Summary and conclusions

Tumour hypoxia is an essential feature of the malignant phenotype. This thesis has explored novel ways of taking advantage of such a critical phenomenon, looking at (1) hypoxia-regulated antigens, (2) the combined effects of anti-angiogenic and T cell immunotherapy, and (3) the chemical induction of hypoxia in tumour cell lines.

Hypoxia-regulated proteins represent novel overexpressed tumour antigens that potentially could be exploited with specific T cell immunotherapy. The findings of Chapter 3 using a hypoxia-regulated tumour antigen  $\beta$ gal in a murine melanoma model, have demonstrated specific T cell responses *in vitro* commensurate with the degree of hypoxic induction in the target cell. This has correlated with anti-tumour effects *in vivo* using both subcutaneous tumour implants and intravenous pulmonary metastases. Notwithstanding, protection against tumour growth in both scenarios was incomplete and inadequate. Various reasons for the tumour escape have been cited and discussed. The issue of effective T cell migration into the tumour mass, and in particular, hypoxic areas of the tumour is pertinent. Further analysis of tumour infiltrative T cells with respect to hypoxic tumour regions is warranted.

Chapters 4 and 5 described the induction and monitoring of a hypoxia-regulated antigen CA9 specific T cell responses in a transgenic mouse model, healthy human HLA-A2 donors and RCC patients. Murine CTL generated *in vitro* and *in vivo* specific for the CA9<sub>254-262</sub> peptide were used as effectors against human target cells. The lack of murine CD8 binding to the human  $\alpha$ 3 domain in TCR-pMHC interactions was possibly

responsible for the reduced avidity of these CTL. The targeting of tumour antigen CA9 in humans was restricted by the low avidity of CTL, being reactive to what essentially was a self antigen. In fact, this problem extends across most immunotherapeutic strategies aimed at inducing effective T cell responses *in vivo*. The lack of correct processing and presentation by antigen presenting cells (APC) was another possibility. The recent emergence of peptide/MHC specific Fab antibodies could allow determination of the correct peptide presented on the surface of the APC (Denkberg et al., 2002). The discrepancy in CTL responses to the CA9<sub>26-35</sub> epitope in RCC patients versus healthy controls could relate to a tumour escape phenomenon.

Chapters 6 and 7 propose two novel methods of induction of hypoxia-regulated antigens. The metronomic dosing of cyclophosphamide was shown to synergise with specific T cell immunotherapy in a murine melanoma model. The combination of the anti-angiogenic effects of metronomic dosing and specific T cell immunotherapy against a hypoxia-regulated antigen remains to be explored. Interestingly, cyclophosphamide has recently been proposed to augment T cell responses by removal of T regulatory cell suppression, an effect that may prove useful in reversing peripheral tolerance to tumour antigens (Ghiringhelli et al., 2004). The use of chemical means to induce expression of hypoxia-regulated proteins was demonstrated with a novel iron chelator desferri-exochelin. Additionally, non-HIF related effects by way of tumour apoptosis and anti-angiogenesis, make iron chelation therapy in combination with immunotherapy yet another therapeutic possibility.

In conclusion, the targeting of hypoxia-regulated tumour antigens *in vitro* and *in vivo* in a murine model has been demonstrated, but is not completely effective in containing tumour growth and expansion. The translation of this data into human systems is hampered by the lack of high avidity T cells reactive to a self-antigen, as well as incorrect processing and presentation by antigen presenting cells. These low avidity interactions between effectors and targets might however, be overcome via augmentation of target antigen expression, by mechanisms that further induce hypoxia in the tumour mass through anti-angiogenic strategies, or through non-hypoxia mediated chemical means as iron chelation therapy. The targeting of the hypoxic tumour phenotype with immunotherapy in synergy with other strategies presents a novel and challenging field of therapeutic possibilities.

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**Abbreviations:**

|                 |                                                  |
|-----------------|--------------------------------------------------|
| $\alpha$ GalCer | $\alpha$ -galactosylceramide                     |
| ADCC            | antibody dependent cell mediated cytotoxicity    |
| APC             | antigen presenting cell                          |
| BMDC            | bone marrow derived dendritic cells              |
| $\beta$ 2m      | $\beta$ -2-microglobulin                         |
| CMV             | cytomegalovirus                                  |
| CT              | Computerised tomography                          |
| CTL             | cytotoxic T lymphocyte                           |
| CTX             | cyclophosphamide                                 |
| DC              | dendritic cell                                   |
| EBV             | Epstein Barr virus                               |
| ELISA           | enzyme linked immuno-sorbent assay               |
| ELIspot         | enzyme linked immuno-spot assay                  |
| FACS            | fluorescence activated cell sorting              |
| GM-CSF          | granulocyte-macrophage colony stimulating factor |
| HIF             | hypoxia inducible factor                         |
| IFN $\gamma$    | interferon $\gamma$                              |
| IFA             | incomplete Freund's adjuvant                     |
| ip              | intraperitoneal                                  |
| iv              | intravenous                                      |
| LCL             | lymphoblastoid B cell line                       |
| MCA             | methylcholanthrene                               |
| MHC I/II        | major histocompatibility class I/II              |
| Mcf             | mean channel fluorescence                        |
| MRI             | magnetic resonance imaging                       |
| MTD             | maximum tolerated dose                           |
| MOI             | multiplicity of infection                        |
| NK              | natural killer                                   |
| NKT             | natural killer -T                                |
| pMHC            | peptide MHC complex                              |
| PBS             | phosphate buffered saline                        |
| pfu             | plaque forming units                             |
| PCR             | polymerase chain reaction                        |
| PBMC            | peripheral blood mononuclear cells               |
| PBL             | peripheral blood lymphocytes                     |
| PHA             | phytohemagglutinin                               |
| RCC             | renal cell carcinoma                             |
| RAG             | recombinase activating gene                      |
| sc              | subcutaneous                                     |
| TCR             | T cell receptor                                  |
| TAA             | tumour associated antigen                        |
| TIL             | tumour infiltrating lymphocytes                  |
| VHL             | von Hippel-Lindau                                |

## Acknowledgments :

My sincere thanks to:

- Enzo and Adrian; it was a privilege to have been supervised by you
- Val and Amanda; for 'babysitting' a bewildered and befuddled Urologist new to the lab
- Dr David Cranston; for the support in my research, and the opportunity to watch a Master Surgeon at work
- Dr Andy Protheroe; for your help and encouragement, and for sharing ideas on tumour immunology
- Ms Karena Le Monnier; for helping procure samples
- Dr David Davies; for your valuable time spent with me over the histology sections
- Dr Helen Turley; for much assistance with immunohistochemistry
- Prof Foo and Chris Cheng from my own Department of Urology, Singapore General Hospital; and the National Medical Research Council of Singapore for financial support

My old friends from the Growth Factor group before I 'jumped ship' into Immunology; John, Uli, Veronica, Heidi, Helen, Kevin Turner (my fellow 'plumber'), Charlie Wykoff (you Da Man!)

And not least, all the great friends and colleagues from the Cerundolo group that I have had the privilege and pleasure to work/play/drink/complain/dream/waste-time-over-coffee with; Mike ('Immunity' beckons), Mao (ciao bella!), Corinna (carpe diem!), KL (see you back home), Vicki (bonza you beaut!), Sarah (the FOXy lady), Sameera, Joelle, Ed Choi (keep dancing man), Pru, Thomas, Caroline, Rod. And from the congenial level 5 side; Uzi (shalom Rabbi!), Kevin (keep up the Mandarin), Rachel, Dawn (my tetramer guru), Andrea (see you in Barbados ☺ ), Jili (Dong Fang Bu Bai), Fareed (viva Brazil!), Jon (you cool trumpet-playing dude), Haja and my good buddy Ian (looking forward to the great NZ-Singapore Institute of Immunology and Sailing/windsurfing).

And cheers to my drinking mates at the Social Club; FACS analysis would not have been quite so enjoyable without a pint (or 2 or 3 or whatever) beforehand ☺ (hic)



This thesis is dedicated to  
*Eunjeong*

And to the memory of  
*Suzanna Paul and David Khoo*

*Your life and spirit remain my inspiration*



