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Combining chemotherapy with immunotherapy to treat mesothelioma: an investigation into the role of CD4+ T cells in a murine model.

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Abstract

Cytotoxic chemotherapy remains the mainstay of treatment for patients with cancer, however, immunotherapy is starting to emerge as an additional modality of treatment. Evidence suggests that chemotherapy can synergise with immunotherapy to improve responses.

Although CD8 T cells have been regarded as the main anti-tumour effector cell, the role of CD4 T cells in orchestrating CD8 and other anti-tumour responses is increasingly recognised. However, the CD4 T cell population contains effector and suppressive subsets with diverse and opposing functions.

This thesis describes the establishment of a murine mesothelioma model with which to study the effects of different CD4 subsets on anti-tumour immune responses, and investigate their capacity to provide cognate help to tumour antigen specific CD8 T cells. Haemagluttin (HA) specific CD4 T cells from transgenic mice were polarised in vitro into Th1, Th2, Th17 and Treg subsets and adoptively transferred alongside HA specific CD8 T cells into mice bearing HA expressing tumours derived from a mesothelioma cell line. The effects of the different CD4 subtypes on tumour growth and their capacity to provide ‘help’ to CD8 T cells was investigated in a prophylactic treatment model and in the context of treatment with gemcitabine chemotherapy.

Results showed that survival and behaviour of in vitro differentiated CD4 subtypes after adoptive transfer was highly variable and that only Th1s displayed anti-tumour activity when injected prophylactically, prior to tumour inoculation. Cytotoxic chemotherapy did not provide a favourable environment for adoptive transfer of in vitro differentiated CD4 cells. No antitumour activity was seen against established
tumours, which may have been due to overriding tumour induced immunosuppressive mechanisms. Successful treatment of established tumours that had been treated with chemotherapy required both the provision of HA specific CD8 cells and the prior removal of an established, endogenous regulatory CD4 T cell population.

Publications arising from this work


- This paper forms the basis of section 1.1
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1 Introduction
1.1 Overview of T cell anti-tumour immune responses

Although anti-cancer immunity involves both the innate and adaptive immune system, CD8+ cytotoxic T lymphocytes (CTL) are one of the most important anti-tumour effector cells (Kennedy and Celis 2008). The T cell immune response can be broken down into the following steps, all of which need to be fulfilled for effective anti-tumour CTL to be generated (Lake and Robinson 2005): i) Tumour antigen(s) must be present, and ii) these must be presented in a context which is seen as dangerous by the immune system; iii) antigens must be acquired and presented by antigen presenting cells (APC) in the draining lymph node; iv) specific T cells must then recognise and respond to tumour antigen by proliferating, exiting the lymph node, recirculating and entering the tumour as cytotoxic T lymphocytes (CTL); and v) once within the tumour they need to overcome the local immunosuppressive environment before they can kill tumour cells. In addition memory cells may need to be generated to produce a sustained response.

It is clear that a growing tumour has managed to escape this process. Failure of the anti-tumour immune response can occur at one or more of these steps. Targeting rate-limiting steps with therapies designed to boost the immune response can improve anti-tumour immunity (Yuan, Gnjatic et al. 2008). In addition to specifically targeted immune therapies, it is also now clear that many traditional cancer therapies can improve key aspects of anti-cancer immunity by inducing tumour cell death in a way that is immuno-stimulatory or by modulating tumour induced immunosuppression (Nowak, Lake et al. 2006).
Tumour antigens – what the host might ‘see’ as being different to self.

Tumours typically express two types of antigen: neo-antigens and self antigens. Neo-antigens (tumour specific antigens) are derived from mutated self proteins or oncogenic viruses, and are not expressed in normal tissue. Malignant cells express numerous neo-antigens as a result of genomic instability (Tomlinson, Sasieni et al. 2002; Weir, Woo et al. 2007; Srivastava and Srivastava 2009). Most of these mutations do not have functional significance for the tumour cell but may still provide potential antigenic targets for immune cells. However, oncogenic mutations in genes which drive tumour cell replication make more attractive targets for immunotherapy as immune escape through loss of gene expression should be incompatible with continued tumour growth.

In addition, tumours can also express normal self proteins, but in abnormal quantities or locations (tumour associated antigens - TAAs)(Schietinger, Philip et al. 2008). TAAs include cancer-testis antigens, for example MAGE and ESO; differentiation antigens, such as tyrosinase, which are also expressed in the tissue of origin; oncofetal antigens such as aFP and CEA; or over-expressed proteins such as Her2 or wild type p53 (Antonia, Mirza et al. 2006; Cloosen, Arnold et al. 2007; Bioley, Dousset et al. 2009; van der Bruggen 2009).

During T cell development, T cell precursors with a strongly self-reactive T cell receptor (TCR) are deleted in the thymus, resulting in a T cell repertoire with a high affinity for foreign antigens and a weak affinity for self antigens. In addition, self reactive T cells can be tolerised peripherally following encounter with antigen in the absence of an activating stimulus and through the actions of regulatory cells. Since tumour neo-antigens are ‘foreign’, in so much as they are not expressed in the thymus, T cell
precursors with high affinity for these antigens escape thymic deletion. In a similar fashion, tumour antigens of the cancer-testis group are largely hidden from developing immune cells. In contrast, most other tumour associated antigens are expressed in other tissues, including the thymus (Cloosen, Arnold et al. 2007), resulting in a lymphocyte population that is either deleted or has low affinity for these antigens.

Multiple tumour antigens generate a hierarchy of T cell responses, with dominant antigens producing stronger responses than sub-dominant or cryptic antigens (Wortzel, Urban et al. 1983; Nelson, Bundell et al. 2000; Bundell, Jackaman et al. 2006). Tumour specific neo-antigens are dominant over antigens shared with normal tissues (Lennerz, Fatho et al. 2005). CTL responses can be generated to weaker antigens, but require higher antigen concentrations and prolonged duration of exposure (Nelson, Bundell et al. 2000). Furthermore, patients with differing HLA haplotypes may generate variable immune responses to the same tumour antigens or tumour vaccines due to differing affinity of the antigen for HLA molecules (Bioley, Guillaume et al. 2009; Bioley, Guillaume et al. 2009).

Although there is a stronger T cell repertoire for tumour neo-antigens, the expression of mutated gene products is usually specific to individual tumours, limiting the general applicability of immunotherapy directed at these antigens. Exceptions include genes such as ras, which mutate in a small number of predictable sites and may, therefore, generate epitopes that are shared between patients (Linard, Bezieau et al. 2002).

Pleasance et al sequenced the genome of small cell lung cancer (Pleasance, Stephens et al. 2009) and melanoma (Pleasance, Cheetham et al. 2009) and identified signature mutational patterns of tobacco smoke and ultraviolet radiation respectively. In the three lung cancer cell lines studied, mutations in CHD7, a chromatin remodeller and
potential oncogene, were found, suggesting that this technique could potentially identify other neo-antigens shared between patients.

In contrast, tumour associated self antigens are more likely to be shared between patients and cancer vaccine strategies to date have mainly focussed on TAAs due to their broader applicability. However, the T cell repertoire for tumour associated antigens is often weak (Cloosen, Arnold et al. 2007) and TAA targeted immunotherapy can cross react with normal tissues, inducing autoimmunity (Gilboa 2001; Dudley, Wunderlich et al. 2002).

*An intact tumor as ‘its own vaccine’*

Generating a strong, tumour specific response with treatments which are applicable to large patient groups is a challenge for vaccine-based immunotherapy and, to date, it has not been feasible to identify neo-antigens at an individual level. To overcome this, immunotherapies have used autologous tumour vaccines, or autologous mRNA gene transfer, to circumvent the need to identify antigens. Individual gene expression profiling and HLA typing is an alternative approach, but is time consuming, costly, and difficult to apply in routine clinical practice. Whilst the above strategies require adequate, accessible tumour tissue, using the in-situ tumour as an antigenic source is an approach that does not require tissue manipulation. Inducing tumour cell death through cytotoxic chemotherapy, radiotherapy or locally administered therapies may alter the amount or the way that tumour antigens engage with the immune system, through exposure of hidden antigens, increased exposure of antigens present at low concentration, or modification of low-affinity antigens. In this way, we can use tumour antigens to prime a more effective immune response without ever knowing their identities: in effect the tumour acts as its own vaccine (van der Most, Currie et al.)
2006; Jackaman, Lew et al. 2008). Studies using chemotherapy to load tumor antigens have confirmed the feasibility of this notion (Nowak, Lake et al. 2003).

**Danger – how tumour cell death can induce an immune response**

The immune system has evolved to ask two main questions of any invading organism – is it *different* and is it *dangerous*. Almost all vaccines harness those two features (antigen plus adjuvant); a key aim of tumour immunotherapy is to kill tumor cells in ways that make the death look dangerous to the host.

Presentation of tumour antigens on MHC class I molecules by antigen presenting cells such as dendritic cells is necessary to prime CD8+ cytotoxic T lymphocytes (CTL). Alone, however, this is insufficient to generate functional effector T cells, and may in fact induce tolerance. Antigens must also be encountered in the context of danger signals, such as those from microorganisms (pathogen associated molecular patterns - PAMPs) or from dying or damaged cells (damage associated molecular patterns - DAMPs) (Matzinger 2002; Lake and Robinson 2005). The mode of cell death is important in determining whether the event is immunogenic or tolerising. For instance, tumour cells treated with alkylating agents induce upregulation of MHC and costimulatory molecules on dendritic cells (DCs), and increase IL-12 secretion when compared to tumour cells killed by freeze-thawing or nucleoside analogues, implicating DNA damage as one potential ‘danger signal’ (Rad, Pollara et al. 2003).

Most chemotherapeutic agents kill tumour cells by inducing apoptosis. Apoptosis is a tightly regulated cellular mechanism which follows the activation of caspases by cell surface death receptors or the release of pro-apoptotic molecules from mitochondria.
Orderly breakdown of cellular and chromosomal structures occurs without disruption of the cell membrane and cellular material is packaged into apoptotic bodies (Edinger and Thompson 2004; Okada and Mak 2004). Apoptotic cell death during normal cell turnover does not induce immune responses, as potential antigens are removed by phagocytosis and are not presented in an immunogenic context. However it is now clear that tumour cell apoptosis is not necessarily an immunologically bland or tolerising event, and under certain circumstances can be immunostimulatory (Restifo 2000; Feng, Zeng et al. 2002). This is dependent on soluble products released from the dying cell and on cell surface signalling molecules. Phosphatidylserine (PS) is one such molecule. Normally restricted to the inner leaflet of the plasma membrane, during apoptosis it crosses to the outer leaflet and provides an ‘eat me’ signal to phagocytic cells. PS promotes the uptake of dying cells by macrophages and induces expression of inhibitory cytokines and suppression of IL-12 production (Kim, Elkon et al. 2004). It has an inhibitory effect on DCs, leading to maturation failure and reduced ability to stimulate CTL and IFN-γ-producing helper T cells (Chen, Doffek et al. 2004), effectively downregulating the immune response. Calreticulin is an endoplasmic reticular protein that translocates to the tumour cell surface within hours of exposure to anthracyclines, also acting as an ‘eat me’ signal to DC well before PS expression and other apoptotic changes are manifest. In contrast to PS mediated DC uptake, cells displaying calreticulin following anthracycline exposure are immunogenic, as evidenced by their ability to act as tumour vaccines and to stimulate IFN-γ production (Chaput, De Botton et al. 2007; Obeid, Tesniere et al. 2007; Panaretakis, Joza et al. 2008; Tesniere, Schlemmer et al. 2009).

Other substances associated with cell damage may be released during apoptosis, and can provide the danger signals required for immunogenic cell death (Skoberne,
As infection provides a key evolutionary pressure for development of immunity, it is not unexpected that innate pathways closely associated with immune response to infection are involved in this activation. During infection, pattern recognition receptors such as toll like receptors (TLRs) recognise pathogen associated molecular patterns (PAMPs) and trigger the production of pro-inflammatory cytokines. Endogenous substances derived from dying tumour cells (DAMPs) can also act on many of these receptors. These include DNA and RNA and their breakdown products (Xiao 2009), heat shock proteins (Feng, Zeng et al. 2002; Javid, MacAry et al. 2007) and the nuclear protein high mobility group box-1 (HMGB1) (Tesniere, Schlemmer et al. 2009). Immune activation by HMGB1 from tumour cells is dependent on TLR4. The clinical relevance of this pathway has been demonstrated in anthracycline-treated breast cancer patients, where the presence of a variant, non-functional TLR4 allele significantly hastened the time to development of metastatic disease (Apetoh, Ghiringhelli et al. 2007).

Uric acid is another inflammatory stimulator associated with cell damage, being produced from purine catabolism during DNA and RNA breakdown. It is released from dying cells, stimulating DC maturation and enhancing cytotoxic CD8 responses to antigen in vivo (Shi, Evans et al. 2003). Similarly, ATP is an intracellular molecule which within the extracellular environment is pro-inflammatory. Cell death caused by a number of chemotherapeutic agents is associated with reduced intracellular and increased extracellular ATP levels (Martins, Tesniere et al. 2009). ATP has a high affinity for the purinergic receptor, P2X7, on DCs, which results in IL-1B secretion. IL-1B plays a role in priming naive CD8 T cells into IFNg producing cells and deficiency of this axis impaired the immune response to oxaliplatin treated tumour cells (Ghiringhelli, Apetoh et al. 2009).
A number of chemotherapy drugs have now been shown to induce tumour cell death in a way which looks dangerous to the immune system (Apetoh, Ghiringhelli et al. 2007; Chaput, De Botton et al. 2007; Obeid, Tesniere et al. 2007; Ghiringhelli, Apetoh et al. 2009; Martins, Tesniere et al. 2009; Tesniere, Schlemmer et al. 2009). In addition, augmenting the anti-tumour immune response through the local administration of agents providing danger signals has an established role in cancer treatment, with historical use of Coley’s toxin and with intravesical BCG for bladder cancer. Tumour regression was observed in mice following local injection of TLR agonists (Currie, van der Most et al. 2008). Combining these local stimuli with systemic immunotherapy can generate systemic immune responses and regression of distal tumours in mice (Broomfield, van der Most et al. 2009).

**Antigen presenting cells – the gateway to immunity or tolerance to tumour antigens**

[Figure 1-1]

Although T cell receptors bind with variable specificity and avidity to self and non-self antigens, T cells themselves do not have the capacity to discriminate dangerous from harmless antigen. Antigen presenting cells, especially dendritic cells, fulfil this crucial role by acquiring antigens within tissues, responding to associated danger signals and subsequently displaying antigen to T cells (signal 1) with the appropriate information about the level of danger present (signal 2). In addition helper (CD4+) T cells recognising antigen presented on MHC class II licence DCs, through co-stimulation, to promote T cell activation. Thus the helper T cell gives a ‘second opinion’ to the DC so that antigens which have previously been seen as dangerous and have hence generated memory responses are promoted as immunogenic.
Figure 1-1  Induction phase of anti-tumour CD8 cytotoxic T cells.

Immature dendritic cells acquire antigen within the tumour and migrate to the draining lymph node. Antigen is processed by the DC and presented to CD4 T cells on MHC class II molecules and cross-presented to CD8 T cells on MHC class I molecules. DC activation is promoted by danger signals, IFNg and ligation of CD40 by helper T cells. Upon activation DCs express co-stimulatory molecules and cytokines, leading to activation of naive tumour antigen specific T cells.

Legends in boxes indicate potential targets for therapeutic intervention.
All nucleated cells express MHC class I molecules and can display endogenously derived antigen bound to MHC class I molecules to CD8 T cells, but only ‘professional’ antigen presenting cells can provide the additional co-stimulation needed to activate naive T cells. Endogenous antigen is generated from self or viral proteins through the actions of proteosomes within the cytosol. It is then transported to the endoplasmic reticulum (ER) in a process which is dependent on the Transporter associated with Antigen Processing (TAP), loaded onto MHC class 1 and trafficked to the plasma membrane. In contrast, exogenous antigens, such as those from tumours, are taken up by antigen presenting cells into endosomes and loaded on MHC class II molecules within the endocytic compartment. CD4 T cells are then able to bind antigen displayed on MHC class II molecules on the cell surface (Guermonprez, Valladeau et al. 2002).

However, some dendritic cells are also able to present exogenous antigens on MHC class I molecules for recognition by CD8 T cells, termed ‘cross presentation’ (Albert, Sauter et al. 1998). Cross presentation of tumour antigen bound to MHC class I is a constitutive feature during many types of tumour growth (Marzo, Lake et al. 1999; Robinson, Lake et al. 1999; Robinson, Scott et al. 2001) even in the absence of any antitumor CTL activity. Cell bound antigen is presented more efficiently than soluble antigen (Li, Davey et al. 2001) and the degree of cross presentation is dependent on the amount of antigen present (Nelson, Bundell et al. 2000). Cross presentation is dependent on TAP, and hence it was initially thought that for cross presentation to occur, following cytosolic degradation by proteosomes, exogenous protein had to join the endogenous pathway in the ER (Brossart and Bevan 1997). However recent work suggests that TAP is present in endosomes and transports antigen destined for cross presentation back into the endosome from the cytoplasm for loading onto MHC I (Burgdorf, Scholz et al. 2008). Interestingly, TAP levels in endosomes are regulated by
the TLR4-MyD88 pathway, implying that TLR danger signals can upregulate signal 1 on
the DC as well as signal 2 (Burgdorf, Scholz et al. 2008).

The fate of the T cell whose T cell receptor binds to cross presented antigen, and
whether it becomes primed or inactivated as result of this encounter, is critically
dependent on the state of maturation of the DC; activated DCs cross prime, whereas
non-activated DCs cross tolerise. Immature DCs are inefficient at cross presenting
antigen and do not express the costimulatory molecules required to activate T cells.
Dendritic cell maturation is initiated by ‘danger signals’ from pathogens or damaged or
dying cells and by inflammatory cytokines, including interferon gamma (Brossart and
Bevan 1997). In addition, ligation of CD40 on dendritic cells is a potent inducer of IL12
secretion and increases their capacity to activate T cells (Cella, Scheidegger et al.
1996). CD40L is usually provided by helper T cells, consistent with the finding that
CD40 activation circumvents the need for CD4 T cell help (Bennett, Carbone et al.
1998; Nowak, Robinson et al. 2003). DC maturation results in increased antigen
uptake, upregulation of MHC expression (Cella, Engering et al. 1997), and expression of
co-stimulators CD80(B7-1) and CD86(B7-2) (Rad, Pollara et al. 2003). During
maturation DCs migrate from tissues to draining lymph nodes, downregulating
endocytic capacity and MHC class II synthesis en route, meaning that antigen
presented to T cells is restricted to that which was internalised at the time any danger
signal was encountered (Guermonprez, Valladeau et al. 2002).

Promoting the CD8 T cell response – overcoming the reluctance of T cells to become
activated and attack tissues.
Priming of CD8 T cells by mature DC in the draining lymph node requires several signals: T cell receptor binding to antigen coupled to MHC class 1, ligation of CD28 on the T cell by CD80 or CD86 on the DC and inflammatory cytokines such as IL-12 and type 1 interferons. However, an effective anti-tumour response also requires that these cells proliferate, survive in the circulation, enter the tumour and fulfil their effector function (Lake and Robinson 2005).

There is evidence that CD4+ cells play a critical role in this process. The addition of tumour antigen specific CD4+ cells to a CD8+ adoptive transfer treatment strategy in mice led to sustained accumulation of tumour specific CD8+ cells in tumour and lymphoid tissues when compared with CD8+ transfer alone. Cytotoxic activity of the CTLs was maintained and mice were protected from tumour growth (Marzo, Kinnear et al. 2000). Persistent CD4+ help and IL-2 secretion are required to maintain CD8 cell function and numbers (Antony, Piccirillo et al. 2005). Direct cell–cell contact from CD4 cells can also protect effector CD8 cells from activation induced cell death (Kennedy and Celis 2006).

Help from CD4 cells during CD8 priming gives rise to CD8 cells that are not only able to function as effector CTL but on restimulation with antigen undergo further clonal expansion leading to the generation of memory cells (Janssen, Droin et al. 2005; Bannard, Kraman et al. 2009; Feau and Schoenberger 2009). This is again dependent on IL-2 secretion (Williams, Tynzink et al. 2006), consistent with the finding that mice cured of tumour through the adoptive transfer of Th1 cells acquired immunological memory whereas those cured by transfer of Th2 cells did not (Nishimura, Nakui et al. 2000). In contrast those CD8 cells that do not receive CD4 help during priming die.
following secondary contact with antigen, partly mediated by upregulation of the TRAIL receptor DR5 (Janssen, Dron et al. 2005).

Although the role of CD4 helper cells in the activation and maintenance of CD8 T cells and the generation of memory is well established, less is known about their precise role following secondary encounter with antigen within the tumour. Because CD4 cells enhance tumour CD8 infiltration (Marzo, Kinnear et al. 2000) it is assumed that through induction of co-stimulation plus cytokine and chemokine production, helper T cells facilitate secondary expansion and survival of CTLs (Kennedy and Celis 2008). In a metastatic murine tumour model, the presence of memory CD4 cells enhanced secondary expansion of memory CD8 cells, increased tumour infiltration of activated CD8 cells, and controlled tumour growth (Hwang, Lukens et al. 2007). However, it is unclear whether this secondary help is mediated directly to CTLs or via further interactions with antigen presenting cells.

**Why does a strong T cell response still fail to eradicate tumours? [Figure 1-2]**

The importance of a robust effector T cell response in mediating successful outcomes to immunotherapy has been recently shown in a clinical human papillomavirus vaccine trial for pre-malignant vulval intraepithelial neoplasia, in which measured T cell responses strongly correlated with regression of lesions (Kenter, Welters et al. 2009). However, in other clinical trials of tumour vaccines against larger, invasive malignancies the effective generation of tumour antigen specific T cells in peripheral blood has not predicted clinical efficacy (Rosenberg, Sherry et al. 2005). This disparity may reflect the weaker activity of T cells generated by vaccines targeting shared self
tumour antigens compared to those directed against viral neo-antigens. It may also reflect the presence of a number of other barriers to effective immunotherapy in established invasive tumours compared to pre-malignant lesions.

**Figure 1-2**  **Effector phase of anti-tumour CD8 cytotoxic T cells**

Cytotoxic CD8 T cells (CTLs) need to exit the circulation and enter the tumour. CD4 cells facilitate tumour infiltration and may promote secondary expansion of CD8s, although it is unclear whether further contact with antigen presenting cells is necessary for this to occur. Following recognition of their cognate antigen presented on MHC class I molecules by tumour cells, CTLs can effect tumour cell killing. Local immunosuppressive mechanisms inhibit the anti-tumour response, including suppression by regulatory cells and inhibitory cytokines, loss of MHC class 1 expression by tumour cells and expression of PD-L1.

Legends in boxes indicate potential targets for therapeutic intervention.
Tumour reactive CTLs may be ineffective because they remain in the periphery or the draining lymph node without actually infiltrating the tumour (Stumbles, Himbeck et al. 2004), or they may disseminate to the tumour but display only weak anti-tumour activity. This suggests that the anti-tumour T cell response can fail at a point downstream of the induction phase.

**Failure of activated T cells to continue to expand and maintain function**

T cell anergy can occur as a result of inadequate costimulation during priming, or can be acquired during later phases of clonal expansion after adequate initial activation (Deeths, Kedl et al. 1999; Mescher, Popescu et al. 2007). Cells that become anergic may be able to fulfil effector functions but are unable to expand further or generate memory cells; this state can be reversed by IL-2, typically supplied by CD4 cells. Acquired anergy provides an inherent brake to the initial rapid CD8 T cell response which will recede after a few rounds of cell division unless further CD4 help is supplied. T cells may also become tolerised following persistent peripheral exposure to antigen, for example, in the setting of a growing tumour. This leads to a failure to proliferate and failure to produce IL-2 in response to antigen although cytotoxic activity may be retained (Tanchot, Guillaume et al. 1998; Ohlen, Kalos et al. 2002). These findings are potentially relevant in human cancer, with Beyer identifying both tumour reactive and non tumour reactive T cell clones coexisting in cancer patients; non reactive cells had not simply been suboptimally activated but had altered molecular programs leading to division arrest anergy (Beyer, Karbach et al. 2009).

**Activated T cells may be switched off by some tumours**
Inhibitory co-receptors, including CTLA4 and PD1, appear to play a major role in inducing and maintaining peripheral T cell tolerance. They are expressed on activated T cells and interact with molecules of the B7 family which are found on APCs but are also expressed by many tumours (Zou and Chen 2008). CTLA-4 is upregulated during T cell activation and causes competitive inhibition of B7-CD28 induced T cell activation, modulates intracellular signalling pathways and leads to decreased IL-2 production, impaired TCR signalling and cell cycle arrest, particularly in the early post-activation phase (Hodi 2007). Anti CTLA-4 treatment has been trialled in melanoma and other cancers with evidence of clinical efficacy (Wolchok and Saenger 2008; Yuan, Gnjatic et al. 2008) and has recently been granted FDA approval for treatment of advanced melanoma (Hodi, O'Day et al. 2010).

The expression of PD-L1 (B7-H1), a ligand for PD-1, is upregulated by IFNg (Blank, Brown et al. 2004) and has been observed in many tumour types, often being associated with a poor prognosis (Zou and Chen 2008). PD-1/PD-L1 interactions impair antitumour T cell responses in mice, which is reversed in PD-1 deficient mice or by blocking PD-1 (Blank, Brown et al. 2004). Mechanistically, expression of PD-L1 by tumours induces T cell apoptosis (Dong, Strome et al. 2002), induces production of IL-10, and may mediate T reg suppressive activity (Zou and Chen 2008)). PD-1/PD-L1 inhibition strategies in patients with cancer are currently in early phase clinical trials. Although both CTLA-4 and PD-1 induced T cell tolerance in an autoimmune diabetes model, only PD-1 was able to maintain tolerance after induction (Fife, Guleria et al. 2006). It is unclear whether this observation will be important in tumour models or clinical testing.

*Suppression by regulatory T cells*
Regulatory T cells (Tregs) modulate the immune response and function by
downregulating potentially harmful autoreactive T cells, and Treg defects are
associated with the development of autoimmune disease (Costantino, Baecher-Allan et al. 2008; Poitrasson-Riviere, Bienvenu et al. 2008). Although other types of suppressive cell have been identified, most regulatory T cells are characterised by expression of surface CD4, CD25 and by intracellular foxp3, a transcription factor that mediates many of their inhibitory capabilities (Fontenot, Rasmussen et al. 2005). Tregs can inhibit effector T cell responses during both the induction (Darrasse-Jeze, Bergot et al. 2009) and the effector stage (Huehn, Siegmund et al. 2004; Sarween, Chodos et al. 2004) by a number of mechanisms, such as direct competition for IL-2, ligation of CTLA-4 with CD80 or CD86 on effector T cells, by promoting the development of inhibitory DCs, or through the generation of inhibitory cytokines, TGFβ and IL10 (von Boehmer 2005). The importance of this subset has been demonstrated in vivo, with Treg depletion mediating tumour regression in mice (Onizuka, Tawara et al. 1999; Rudge, Barrett et al. 2007).

Increased Treg infiltration has been demonstrated in many human tumour types, usually associated with decreased anti-tumour immune responses and worse prognosis (reviewed in (Beyer and Schultze 2006)). However, Treg accumulation is not a universal feature of human cancer, implying a lack of immune response to the tumour or other means of regulation such as myeloid derived suppressor cells.

Furthermore, in colorectal cancer, two independent cohorts demonstrated improved survival in patients with a high frequency of tumour infiltrating Tregs (Frey, Droeser et al. 2009; Salama, Phillips et al. 2009). One explanation is that this finding actually reflects increased immunogenic stimuli within these tumours - either due to tumour antigens or due to gut pathogens - and a subsequent robust immune response.
The prognostic significance of Tregs in many human cancers, together with the success of Treg depletion in murine tumours, suggests Treg depletion may work as a clinical therapeutic strategy. This has been attempted through anti CD25 therapies, which have shown effective Treg depletion and enhanced CTL response to subsequent peptide vaccination (Rech and Vonderheide 2009). However, a different Treg depletion strategy using the cytotoxic agent cyclophosphamide may be more readily translatable to the clinic. At high doses cyclophosphamide is cytotoxic and causes immunosuppression but at low doses it preferentially depletes numbers of Tregs (Ghiringhelli, Larmonier et al. 2004; Ghiringhelli, Menard et al. 2007; van der Most, Currie et al. 2009) and impairs Treg function (Lutsiak, Semnani et al. 2005). A single dose of cyclophosphamide depleted Tregs and when followed by an immunotherapy cured mice with established tumours (Ghiringhelli, Larmonier et al. 2004). In humans low dose oral cyclophosphamide in patients with advanced cancer selectively depleted the Treg subset and enhanced the cytotoxic capacity of T and NK cells (Ghiringhelli, Menard et al. 2007).

*Immune escape within the tumour microenvironment*

Cytotoxic T cells recognise antigen bound to MHC class 1. However reduced expression of MHC class 1, usually due to epigenetic regulation of TAP expression, has been observed in many tumour types and associated with a poor prognosis in patients with colorectal cancer (Watson, Ramage et al. 2006). ‘Darwinian’ selection of these resistant clones through intrinsic or therapeutic immune pressure may explain why some patients who initially respond to immunotherapy then subsequently relapse (Restifo, Marincola et al. 1996).
Changes in expression patterns of non-antigenic molecules may also alter traffic to the tumour. Villablanca et al showed that human and mouse tumours can subvert the migratory ability of mature dendritic cells by the expression of LXRα ligands (Villablanca, Raccosta et al. 2010). LXRαs bind to oxidised cholesterol and when activated on dendritic cells inhibited expression of CCR7, a chemokine required for DC migration to the draining lymph node. This resulted in impaired DC migration to draining lymph nodes, reduced T cell priming and impaired antitumour activity.

Immunosuppressive cytokines within the tumour also impair immune responses and transforming growth factor beta (TGFβ) has been implicated in many different types of cancer (Elliott and Blobe 2005). TGFβ induces expansion of Treg (Ghiringhelli, Puig et al. 2005) and inhibits T cell effector function (Ahmadzadeh and Rosenberg 2005). Interventions targeting TGFβ have been shown to reduce tumour growth in vivo, and to enhance the effectiveness of other immunotherapies (Marzo, Fitzpatrick et al. 1997; Kim, Buchlis et al. 2008). Metabolic dysregulation also contributes to local immunosuppression. The enzyme indolamine 2,3-dioxygenase (IDO) is expressed in numerous cancers (Uyttenhove, Pilotte et al. 2003). It is upregulated by IFNγ and causes tryptophan breakdown which leads to T cell apoptosis (Lob, Konigsrainer et al. 2009). Intervention aimed at silencing IDO expression have shown antitumour activity in a mouse melanoma model (Zheng, Koropatnick et al. 2006).
1.2 Helper T cell subsets and their role in anti-tumour immunity

1.2.1 CD4 subtypes and differentiation

The ability of a subset of T cells expressing the marker CD4 to help promote CD8+ cytotoxic T lymphocyte responses has been known for over 30 years (Keene and Forman 1982). CD4+ T cells recognise antigen presented on MHC class II complexes by antigen presenting cells, such as dendritic cells, macrophages and B cells. CD4+ helper T lymphocytes can further differentiate into distinct subsets which have diverse effects on immunological function, mediated through the secretion of characteristic sets of cytokines. The prototypic division of Th subsets was into Th1s, which produce IFNg, TNFa, IL-2 and promote inflammatory responses and cell mediated immunity, and Th2s, which secrete IL-4, IL-5, IL-13 and promote humoral responses (Mosmann, Cherwinski et al. 1986). Recently Th17s, an IL-17 secreting CD4+ subset, have been identified (Harrington, Hatton et al. 2005; Park, Li et al. 2005). Th17s appear to be involved in tissue responses to extracellular pathogens and have been implicated in many autoimmune diseases. Th17s are found in highest concentration in lung and digestive mucosa.

Activation of naive CD4+ T cells requires that T cell receptor on the CD4 cell binds to antigen presented by MHC class II on antigen presenting cells (signal 1) and that sufficient co-stimulation (signal 2) is provided, such as ligation of CD28 on the T cell by CD80/86 on the antigen presenting cell. The subsequent differentiation of activated CD4+ cells into Th1, Th2 and Th17 subtypes is dependent on the presence of certain cytokines at the time of activation.
Th1 cell differentiation is induced by IL-12. IL-12 is produced by activated dendritic cells (Cella, Scheidegger et al. 1996) and acts through the IL-12 receptor to drive STAT-4 mediated activation of the Th1 transcription factor Tbet. Interferon gamma can also act in an autocrine manner to further promote expression of Tbet through STAT-1 (Kaiko, Horvat et al. 2008).

Th2 differentiation is primarily driven by IL-4 which promotes expression of the transcription factor GATA-3 through STAT-6. Other, non-cytokine related factors can also influence Th1/Th2 differentiation including the strength of TCR stimulus; with low level stimulation insufficient to induce differentiation, moderate stimulation favouring Th2 and a strong stimulus favouring Th1 differentiation (Rogers and Croft 1999).

Th17 differentiation can be induced by a combination of IL-6 and TGFb. Furthermore, IL-21 is produced by Th17 cells and can itself, in combination with TGFb, induce Th17 cells. In humans, IL-1B plus TGFb may also be able to induce Th17s. Additionally, IL-23 is important for the survival and activation of Th17 cells, although is not required for differentiation from naive T cells (Harrington, Hatton et al. 2005; Park, Li et al. 2005; Korn, Bettelli et al. 2009). Following activation of CD4 cells in the presence of the necessary Th17 inducing cytokines, expression of ROR gamma is upregulated. ROR gamma is required and sufficient for the expression of IL-17 and hence is the defining transcription factor for this lineage (Ivanov, McKenzie et al. 2006).

Cytokines produced by helper subsets have antagonistic effects on the differentiation of other subsets, ensuring that once established, the polarity of the CD4 immune response is reinforced. IFNg produced by Th1s antagonises Th2 development and conversely IL-4 antagonises Th1 differentiation (Gajewski and Fitch 1988; Chen and Liu
2009). Th17 differentiation is inhibited both by IL-4 and IFNγ (Harrington, Hatton et al. 2005; Park, Li et al. 2005).

Regulatory CD4+foxp3+ T cells can arise naturally in the thymus (nTreg), or may be converted peripherally from CD4+foxp3- cells (inducible Treg, iTreg)(Apostolou, Sarukhan et al. 2002; Apostolou and von Boehmer 2004). Generation of iTregs in vivo and in vitro requires TCR stimulation, TGFβ and IL-2 (Chen, Jin et al. 2003; Curotto de Lafaille and Lafaille 2009; Long, Rieck et al. 2011). Both nTreg and iTreg constitutively express CD25, GITR and CTLA-4, and mediate suppressive functions in a contact dependent manner. However the nTreg and iTreg populations may have differing roles as a result of their TCR repertoires. nTregs are generated by high avidity interactions in the thymus with organ specific self antigens (Apostolou, Sarukhan et al. 2002). iTregs can be generated in the periphery from naive CD4 cells through contact with endogenously expressed or exogenously administered antigens (Apostolou and von Boehmer 2004; Knoechel, Lohr et al. 2005) and as a consequence share a repertoire with the naive T cell population. Therefore, it has been proposed that the role of nTregs is to prevent autoimmunity to self antigens whereas iTregs maintain tolerance at sites of foreign antigen, such as the gut (Curotto de Lafaille and Lafaille 2009). Since tumours express both self and foreign antigens this concept has relevance for tumour immunity. However, it is unclear what proportion of Tregs react with specificity to tumour antigens (Wang, Peng et al. 2005), or whether they are recruited through the recognition of shared self antigens that are co-expressed by tumour cells (Nishikawa, Kato et al. 2003; Darrasse-Jeze, Bergot et al. 2009).
1.2.2 Plasticity between CD4 subsets

Despite the identification of distinct CD4 subtypes - Th1, Th2, Th17 and Treg - distinguishable by different cytokine secretion profiles and expression of specific transcription factors, it has long been recognised that a degree of plasticity exists between these lineages (Murphy and Stockinger 2010).

Th1 and Th2 cells can be converted into the other subtype in the early stages of activation but not after prolonged stimulation (Murphy, Shibuya et al. 1996), suggesting that the number of rounds of cell division may impact on phenotypic stability. Furthermore, the stability of Th1s and Th2s appears greater than that of Th17s and Tregs. The differentiation pathways of both Th1 and Th2s contain positive feedback loops which reinforce expression of the transcription factors tbet and GATA-3 respectively (Ouyang, Lohning et al. 2000; Mullen, High et al. 2001), providing phenotypic stability to the lineage. In contrast, no reinforcing transcriptional circuits have been identified for RORγ in Th17s or foxp3 in Tregs.

Both Th17s and inducible Tregs share common features in their differentiation pathway. TGFβ induces the expression of both RORγ and foxp3, however the additional presence of inflammatory cytokines IL-6, IL-21 or IL-23 suppresses foxp3 expression and enhances Th17 development (Bettelli, Carrier et al. 2006; Nurieva, Yang et al. 2007; Zhou, Lopes et al. 2008). In contrast high levels of TGFβ favour foxp3 mediated suppression of RORγ and induces Treg development (Zhou, Lopes et al. 2008). IL-2 further enhances Treg differentiation and inhibits Th17 generation (Kryczek, Wei et al. 2007). As such the balance between Th17 and Treg differentiation is influenced by local concentrations of inflammatory cytokines and TGFβ.
IFNγ/IL-17 double producing cells are detectable in patients with chronic inflammatory conditions suggesting a possible relationship between Th1 and Th17 lineages (Boniface, Blumenschein et al. 2010). Th17s convert into Th1s after exposure to IL-12 and this is dependent on STAT-4/ tbet (Lee, Turner et al. 2009). In vitro differentiated Th17s also converted to IFNγ+ ‘Th1 like’ cells in lymphopenic environments (Nurieva, Yang et al. 2009). In another model, Th17 cells generated in vitro were readily converted into Th1s or Th2s by IL-12 or Il-4 respectively, however this was not the case for Th17 cells found in vivo, which maintained IL-17 expression despite culture ex vivo in these conditions (Lexberg, Taubner et al. 2008). This suggests that In vitro differentiation conditions do not necessarily replicate those found in vivo. A novel reporter mouse strain allowing fate tracking of cells which had previously expressed IL-17 enabled Hirota et al to show that during experimental autoimmune encephalitis, inflammation was mostly mediated by cells which had produced IL-17 before their conversion to IFNγ+ cells by IL-23 (Hirota, Duarte et al. 2011). This only occurred under certain inflammatory stimuli, suggesting that the fate of Th17s is determined by distinct inflammatory conditions which drive Th17 plasticity into an IFNg secreting pro-inflammatory effector cell.

### 1.2.3 CD4 helper subtypes and anti-tumour responses

CD4 cells provide help during both the induction and effector phase of the CD8 T cell response. Ligation of CD40L on DCs with CD40 on CD4 cells provides an activating signal to the DC, stimulating the expression of costimulatory molecules, MHC expression and IL-12(Cella, Scheidegger et al. 1996). Th cells also secrete cytokines
such as IL-2, important for the generation and survival of cytotoxic CD8s (Antony, Piccirillo et al. 2005) and for memory responses (Janssen, Droin et al. 2005). Additionally CD4 helper T cells can promote anti-tumour responses mediated by innate immune cells, including macrophages, NK cells and granulocytes (Hung, Hayashi et al. 1998; van den Broeke, Daschbach et al. 2003; Perez-Diez, Joncker et al. 2007; Heusinkveld, de Vos van Steenwijk et al. 2011). CD4 cells can also exhibit direct cytotoxicity against tumour cells that express MHC class II molecules as a result of exposure to IFNg (Quezada, Simpson et al. 2010; Xie, Akpinarli et al. 2010).

The role of regulatory T cells in suppressing anti-tumour immunity is well established and is discussed in section 1.1. However the different ways that effector subsets influence anti-tumour immune responses is less clear. Th1 cells have traditionally been thought to be the most important helper T cell in the context of anti-tumour immunity. Rejection of tumour in a murine model was shown to correlate with a Th1 response whereas tumour progression was associated with Th2 responses (Hamilton and Bretscher 2008). Secretion of the Th1 cytokine IFNg has been found to be necessary for successful outcomes to a number of different immunotherapies (Murphy, Welniak et al. 2003; Moeller, Haynes et al. 2005; Muranski, Boni et al. 2008) and for CD4 mediated tumour elimination (Mumberg, Monach et al. 1999) and upregulates expression of MHC molecules by tumour cells (Quezada, Simpson et al. 2010). IFNg produced by Th1 CD4+ cells interacts with innate immune cells, stimulates production of reactive oxygen species by tumoricidal macrophages (Hung, Hayashi et al. 1998), converts tolerogenic M2 macrophages into inflammatory M1 macrophages (Heusinkveld, de Vos van Steenwijk et al. 2011) and is a key cytokine for NKT cell anti-tumour immunity (Hong, Lee et al. 2006). However, although important in initial anti-tumour activity, IFNg has also been shown to impair T cell memory formation and
secondary immune responses to a later tumour rechallenge through induction of CD4 T cell apoptosis (Berner, Liu et al. 2007). It also upregulated expression of PD-L1 in squamous cell carcinomas and has negative effects on Th17 cells, suggesting that the balance between anti-tumour and tumour-promoting effects may be complex (Tsushima, Tanaka et al. 2006; Chen and Liu 2009).

In contrast, an increased intratumoural Th2 infiltrate predicted a poor prognosis in patients with pancreatic cancer (De Monte, Reni et al. 2011). However, despite being considered unhelpful in anti-tumour immune responses, when in vitro differentiated Th2 cells were adoptively transferred into tumour bearing mice, they mediated tumour rejection as effectively as Th1s through a CD8 dependent mechanism (Nishimura, Iwakabe et al. 1999). However, only mice that received Th1 cells developed immunological memory and resisted rechallenge with tumour. In another model maximal anti-tumour responses to a whole cell vaccine required both Th1s and Th2s to be present in the tumour microenvironment (Hung, Hayashi et al. 1998). The Th2 component of this response was mediated by IL-4 dependent recruitment of eosinophils to the tumour site, suggesting that eosinophils may a Th2 anti-tumour effector cell.

Th17 cells normally constitute a small fraction of the CD4+ T cell population, but increased concentrations of Th17 cells have been found in the tumour microenvironment (Miyahara, Odunsi et al. 2008; Kryczek, Banerjee et al. 2009; Zhang, Yan et al. 2009) and the peripheral blood (Koyama, Kagamu et al. 2008; Derhovanessian, Adams et al. 2009; Horlock, Stott et al. 2009) of patients with a diverse range of cancers. Their role in tumour responses is still controversial. In hepatocellular carcinoma, tumour infiltration of Th17s correlated with poor prognosis
and with increased tumour angiogenesis (Zhang, Yan et al. 2009). This is consistent with experimental models showing that IL-17 promotes angiogenesis and tumour growth (Numasaki, Fukushi et al. 2003; Wang, Yi et al. 2009). However in 201 ovarian cancer patients, tumour infiltrating Th17s correlated positively with the presence of effector cells and effector cytokines and were inversely associated with regulatory cells (Kryczek, Banerjee et al. 2009). High levels of IL-17 in ascitic fluid predicted improved survival in this cohort. Increased Th17 frequencies in peripheral blood has been associated with less advanced disease in small cell lung cancer (Koyama, Kagamu et al. 2008) and with improved responses to treatment in prostate and breast cancer (Derhovanessian, Adams et al. 2009; Horlock, Stott et al. 2009).

In animal models adoptive transfer of in vitro differentiated tumour antigen specific Th17 cells into lymphopenic (Muranski, Boni et al. 2008) and non-lymphopenic mice (Martin-Orozco, Muranski et al. 2009) has been shown to cause tumour rejection more effectively than in vitro differentiated Th1 cells. The presence of lymphopenia appears to alter the behaviour of these cells following transfer. In lymphopenic environments Th17s convert into IFNg producing ‘Th1 like’ cells but this does not occur in normal hosts (Nurieva, Yang et al. 2009). Consistent with this, in lymphopenic mice, tumour eradication was dependent on secretion of IFNg but not on IL-17 (Muranski, Boni et al. 2008), suggesting that in the lymphopenic situation conversion of Th17s into IFNg producing cells is a critical component of their anti-tumour activity. However Th17s have also shown anti-tumour activity in non-lymphopenic mice in a model of metastatic lung melanoma, where the Th17 phenotype was maintained in vivo (Martin-Orozco, Muranski et al. 2009). Th17 cells caused increased DC infiltration of tumour and increased activation of tumour specific CD8s when compared with Th1 cells. This effect was compromised in IL-17 deficient mice but not by IFNg neutralising
antibodies, suggesting that, unlike in the lymphopenic situation, Th17s can mediate anti-tumour activity as a result of IL-17 production.

1.3 The effects of chemotherapy on anti-tumour immunity

Cytotoxic chemotherapy can cause lymphopenia and neutropenia and until recently the notion that chemotherapy could synergise with immunotherapy was not considered plausible. However, it is now clear that chemotherapy can have immunostimulatory effects at a number of different points in the anti-tumour immune response:

Removal of suppressive cells

In causing lymphopenia, chemotherapy depletes regulatory T cells as well as those T cells which have been tolerised to tumour antigens. Depletion of regulatory T cells by cyclophosphamide (Ghiringhelli, Larmonier et al. 2004; Lutsiak, Semnani et al. 2005; Ghiringhelli, Menard et al. 2007; van der Most, Currie et al. 2009) or depletion of myeloid derived suppressor cells with gemcitabine (Suzuki, Kapoor et al. 2005) can enhance antitumour activity through removal of negative regulation. Lymphodepletion in combination with adoptive immunotherapy has shown efficacy in mice (Dummer, Niethammer et al. 2002; Hu, Poehlein et al. 2002) and humans (Dudley, Wunderlich et al. 2002).

Homeostatic proliferation

Following chemotherapy-induced lymphopenia, T cell numbers can either be restored through thymic pathways by active thymopoiesis which produces naive T cells, or
through peripheral expansion of T cell clones, through homeostatic proliferation or antigen driven expansion (Mackall, Hakim et al. 1997). In adults the thymus involutes with age, meaning the thymic pathway becomes compromised and the recovery of the naive T cell repertoire may remain diminished for many years. In contrast homeostatic proliferation results in rapid expansion of the peripheral T cell pool. Although the repertoire of the T cell pool which has regenerated through homeostatic and/or antigen driven proliferation is restricted, this environment may maximise the expansion of tumour reactive clones, improving anti-tumour immunity (Williams, Hakim et al. 2007). Homeostatic proliferation is critically dependent on IL-7 (Tan, Dudl et al. 2001). The IL-7 receptor is expressed at high levels on naive T cells, but is downregulated after activation, and is expressed at a low level on Tregs. When lymphocyte numbers are depleted availability of IL-7 increases and T cells proliferate until IL-7 has been consumed, as illustrated by a dynamic, inverse relationship between serum IL-7 levels and T cell numbers observed in patients recovering from lymphopenia of varying etiology (Fry, Connick et al. 2001). IL-7 administered to patients with melanoma increased circulating CD4 and CD8 T cells and decreased proportions of Tregs (Rosenberg, Sportes et al. 2006). IL-7 also increases T cell trafficking to lymph nodes (Chu, Memon et al. 2004) and provides an anti-apoptotic signal to T cells (Li, Jiang et al. 2004).

**Immunogenic tumour cell death**

Additional mechanisms for immune modulation following chemotherapy include increased antigen release, and upregulation of immunogenic surface molecules. Apoptotic tumour cell death increases the quantity of antigen released and augments cross presentation by mature DCs (Rovere, Sabbadini et al. 1999). Chemotherapy
induced cell death can also be qualitatively immunogenic through upregulation of surface calreticulin (Chaput, De Botton et al. 2007; Obeid, Tesniere et al. 2007; Panaretakis, Joza et al. 2008) or release of intracellular derived ‘danger signals’ (Apetoh, Ghiringhelli et al. 2007; Apetoh, Ghiringhelli et al. 2007; Ghiringhelli, Apetoh et al. 2009; Martins, Tesniere et al. 2009). Treatment with gemcitabine results in increased antigen cross presentation and priming of tumour specific CD8 cells (Nowak, Lake et al. 2003). Chemotherapy can also sensitise those cells not directly lysed by treatment to subsequent killing by immune cells, through upregulation of death receptors Fas (CD95) or TRAIL receptors (DR5) (Mattarollo, Kenna et al. 2006; van der Most, Currie et al. 2009)

1.4 Combined chemo-immunotherapy

The conventional oncology testing field for new treatments is metastatic disease which has failed multiple lines of therapy. Such a high tumour burden with its associated levels of immunosuppression may be a too high a hurdle for immunotherapy alone to achieve clinically significant results. Many murine studies have demonstrated responses to immunotherapy in very early tumours but fewer have demonstrated efficacy against larger, advanced tumours. These very small tumours are unlikely to be representative of most human cancers. However, murine studies have also shown that combining different types of immunotherapy, or combining immunotherapy with chemotherapy, can lead to responses against larger tumours and distal disease.

A number of clinical trials of chemo-immunotherapy have been reported with some promising results, although larger randomised controlled trials are still required (reviewed in (Zitvogel, Apetoh et al. 2008)).

1.5 Adoptive immunotherapy in the treatment of cancer

The potential for adoptively transferred T cells, harvested from tumours and expanded in vitro in IL-2, to mediate tumour regression was first demonstrated in murine models over 25 years ago (Rosenberg, Spiess et al. 1986). Adoptive immunotherapy using tumour infiltrating lymphocytes (T.I.L.) expanded ex vivo and administered with high dose IL-2 has demonstrated reported response rates of 49-72% in advanced metastatic melanoma, with complete response rates of 12-40% (Rosenberg, Yang et al. 2011). Similar response rates were seen when CD8s were enriched from T.I.L. cultures prior to adoptive transfer (Dudley, Gross et al. 2011). Other groups have generated tumour specific CD8 T cell clones through in vitro peptide stimulation of CD8s isolated from peripheral blood and shown that these cells home to tumour sites, with variable clinical response rates observed (Mitchell, Darrah et al. 2002; Yee, Thompson et al. 2002; Mackensen, Meidenbauer et al. 2006). In all these techniques, CD8s were expanded in vitro with IL-2 and activating anti-CD3 antibody to obtain sufficient cell numbers (>1x10^10) for adoptive transfer. Following adoptive transfer, administration of high doses of IL-2 was also required for cells to survive in vivo, traffic to antigen
positive sites and elicit tumour specific responses. In the absence of IL-2, transferred cells did not persist (Mitchell, Darrah et al. 2002; Yee, Thompson et al. 2002).

Although animal models have demonstrated the potential benefit of using CD4 cells for adoptive immunotherapy (Muranski and Restifo 2009; Quezada, Simpson et al. 2010; Xie, Akpinarli et al. 2010), clinical experience of CD4 adoptive immunotherapy in humans is limited, although successful treatment of metastatic melanoma using autologous NY-ESO-1 specific CD4 clones has been reported (Hunder, Wallen et al. 2008). CD4 cells potentially offer benefits for adoptive immunotherapy, not only in providing help for CD8 T cells (Antony, Piccirillo et al. 2005) and through recruitment of innate immunity (Perez-Diez, Joncker et al. 2007) but CD4 cells may also exhibit direct cytotoxicity against tumour cells (Quezada, Simpson et al. 2010). Perez-Diez et al directly compared the anti-tumour activity of adoptively transferred tumour specific CD4 and CD8 T cells against HY antigen expressing tumours and found CD4 cells to be more effective than CD8 cells, an effect which appeared to be due to interactions with NK cells. Furthermore, since CD4 cells recognise antigen presented on MHC class II by antigen presenting cells, their anti-tumour activity is not directly dependent on antigen expression by tumours. Downregulation of MHC class I molecules on tumours does not therefore lead to immune escape from CD4 cells as it does from CD8 T cells (Muranski and Restifo 2009).

Adoptive immunotherapy protocols almost invariably have involved some form of preconditioning regimen to cause lymphodepletion prior to adoptive transfer. Myelosuppressive chemotherapy (Dudley, Wunderlich et al. 2002; Dudley, Wunderlich et al. 2005), total body irradiation (Wrzesinski, Paulos et al. 2011) and combined chemo-radiotherapy (Dudley, Yang et al. 2008) have all been employed to achieve this.
The beneficial effects of lymphodepletion include depletion of regulatory T cells (Antony, Piccirillo et al. 2005; Yu, Lee et al. 2005; Teng, Swann et al. 2010) and myeloid derived suppressor cells (Suzuki, Kapoor et al. 2005). However non-myeloablative preconditioning was found to improve CD8 T cell reactivity even in the genetic absence of regulatory T cells, through removal of endogenous cells responsive to the homeostatic cytokines IL-7 and IL-15, thus increasing their availability for the adoptively transferred cells (Gattinoni, Finkelstein et al. 2005). These observations support the concept that lymphodepletion creates ‘space’ for adoptively transferred tumour reactive T cells to expand (Weber, Atkins et al. 2011). Additionally total body irradiation also causes systemic inflammation and TLR mediated activation of innate immune cells due to translocation of gut microbial flora (Paulos, Wrzesinski et al. 2007). Increasing the intensity of irradiation increases the efficacy of adoptively transferred CD8 T cells in mice (Wrzesinski, Paulos et al. 2011) and humans (Dudley, Yang et al. 2008).

1.6 Chemo-immunotherapy in malignant mesothelioma

Mesothelioma is a malignant tumour of pleural and peritoneal surfaces that is associated with asbestos exposure in the vast majority of cases. The boom in asbestos use during the latter half of the 20th century has lead to an epidemic of asbestos related diseases. A long latency exists between exposure and development of mesothelioma, such that although asbestos usage has been banned in the UK since the 1980s, the incidence of mesothelioma is not expected to peak until 2015 (Hodgson,
Asbestos use is still on the increase in many developing countries and a second global wave of asbestos-related disease is predicted as a result.

Mesothelioma is incurable and responds poorly to currently available treatments. Since mesothelioma grows as multifocal pleural nodules which encase the lung, it is generally not amenable to surgical resection. Most patients present with advanced disease. A combination of pemetrexed and cisplatin is the only treatment regimen proven to be beneficial, although the effects are modest, with a median prolongation of survival of 12 weeks (Vogelzang, Rusthoven et al. 2003). Non-randomised studies have demonstrated individual responses to other agents, including gemcitabine (Nowak, Byrne et al. 2002).

Several other treatment strategies have been tested in early phase clinical trials but with no proven benefit to date, including gene therapy (Sterman, Recio et al. 2007) and inhibition of growth factors and angiogenic pathways (Garland, Rankin et al. 2007). Immunotherapy has been trialled in mesothelioma in both the pre-clinical and the clinical setting. Over 85% of patients with mesothelioma have an associated pleural effusion at the time of presentation, which potentially offers a route for local administration of therapeutic agents. A number of early clinical studies examined the feasibility of intrapleural IL-2 administration, with response rates of up to 50% reported (Astoul, Picat-Joossen et al. 1998). Intrapleural immunotherapy has also been trialled in the form of IFNβ gene transfer using an adenoviral vector, with immune responses detected in 7 of 10 patients and clinical responses in 4 patients (Sterman, Recio et al. 2007). In contrast, intrapleural administration of activated macrophages and IFNγ demonstrated little anti-tumour activity against mesothelioma (Monnet, Breau et al. 2002).
Trials of tumour vaccines have been able to generate demonstrable T cell responses in patients with mesothelioma, but the effects of these interventions on survival are unknown. When an autologous tumour cell lysate was administered intradermally with GM-CSF, 7 of 22 patients developed either cell-mediated or humoral immune responses (Powell, Creaney et al. 2006). More recently, immune responses to a dendritic cell vaccine were detected in 10 of 10 patients and cytotoxicity against tumour cells was seen in 4 of 8 patients (Hegmans, Veltman et al. 2010). Vaccination directed against Wilms tumour 1 (WT1) epitopes, which is highly expressed in mesothelioma, elicited T cell responses in 6 of 9 patients with mesothelioma (Krug, Dao et al. 2010).

The effects of combining chemotherapy with immunotherapy were investigated in a murine model of mesothelioma (Nowak, Robinson et al. 2003) using an antibody which promotes activation of cellular immune responses through ligation of CD40. This study demonstrated the potential for chemotherapy to synergise with immunotherapy and this approach is being translated into early phase clinical trials.

1.7 The AB1-HA mesothelioma model

The tumour cell line used in this model of malignant mesothelioma was generated by injecting mice intraperitoneally with crocidolite asbestos. Around one third of mice develop tumours between 7 and 25 months later and these were confirmed to be mesothelioma on cytology and histology. Tumour cells were harvested from ascites and passaged in vitro and in vivo until stable clonal cell lines were established (Davis, Manning et al. 1992). AB1 was derived from a BALB/c mouse. AB1 is a sarcomatoid
mesothelioma and has morphological and ultrastructural features similar to human mesothelioma on electron microscopy. An inoculation of $5 \times 10^5$ cells produces tumour in 100% of mice.

AB1 cells expressing the influenza antigen haemagglutinin (AB1-HA) have been generated through transfection of AB1 with the HA gene derived from the Mt Sinai strain of the PR8 influenza virus (Marzo, Lake et al. 1999). In this context HA acts as a tumour neo-antigen, but does not alter tumour morphology or MHC expression.

The tumour microenvironment in this model has similarities and differences to that observed in human mesothelioma. MHC class 1 is expressed by both human mesothelioma (Yamada, Oizumi et al. 2010) and AB1 (Leong, Marley et al. 1997). MHC class 2 is not expressed, even after exposure to IFNγ (Leong, Marley et al. 1997). Consistent with this, CD8 T cells have been shown to an important effector cell in human mesothelioma and in the AB1HA model (Rudge, Barrett et al. 2007; Currie, van der Most et al. 2008; Yamada, Oizumi et al. 2010). Both human and Ab1Ha tumours are infiltrated with regulatory T cells (Hegmans, Hemmes et al. 2006; Rudge, Barrett et al. 2007; van der Most, Currie et al. 2009) and myeloid derived suppressive cells (Hegmans, Hemmes et al. 2006; Veltman 2010) and these cells were found to suppress immune responses in the AB1HA model (Rudge, Barrett et al. 2007; van der Most, Currie et al. 2009).

In contrast to most human mesotheliomas and other murine mesothelioma cell lines, AB1 produces little TGFβ (Maeda, Ueki et al. 1994; Kumar-Singh, Weyler et al. 1999; Suzuki, Kapoor et al. 2004). As a result blockade of TGFβ induced immunosuppression has little effect on AB1HA, when compared to TGFβ secreting mesothelioma lines (Suzuki, Kapoor et al. 2004).
The AB1 and AB1HA model have been used extensively by a number of groups to study immune responses to mesothelioma (Nowak, Robinson et al. 2003; Hegmans, Hemmes et al. 2005; Rudge, Barrett et al. 2007; Darrasse-Jeze, Bergot et al. 2009; Bergot and Klatzmann 2010). The presence of HA antigen in this model does not change the growth rate of tumour in nude mice, compared with the parent AB1 line. In wildtype mice however, tumour growth is observed but is delayed in comparison to AB1 (Marzo, Lake et al. 1999), demonstrating that HA antigen does induce an anti-tumour immune response, but that this is insufficient to mediate tumour rejection. Depletion of Tregs led to higher rates of tumour rejection in mice inoculated with AB1HA than those receiving AB1, suggesting that this HA specific immune response is suppressed by regulatory T cells (Bergot and Klatzmann 2010). However mice cured of AB1HA tumours were still able to reject subsequent challenge with AB1, demonstrating that even in the presence of the HA antigen, memory immune responses are generated to other non HA antigens (Nowak, Robinson et al. 2003; Bergot and Klatzmann 2010).

Chemo-immunotherapy, in the form of gemcitabine and anti-CD40 antibody, was equally efficacious in AB1-HA and the parent line AB1, demonstrating that immunological eradication of tumour in this setting was primarily mediated by non-HA mechanisms (Nowak, Robinson et al. 2003).

The advantage of using HA transfected cell lines are that the class I and class II epitopes are known and that anti-HA TCR transgenic mice are available and can be used to examine HA specific immune responses. T cell receptors of CL4 mice are class I restricted and recognise residues PR/8 HA; 518-526 (sequence: IYSTVASSL) (Morgan, Liblau et al. 1996) and thus can be used a source of HA specific CD8 T cells. T cells from HNT mice express a TCR which is class II restricted and recognises the epitope PR/8 HA;
Gemcitabine is used as the chemotherapeutic agent in this model. Gemcitabine (2,2-difluorodeoxycytidine) is a cytidine analogue and a cytotoxic agent used to treat a wide variety of human cancers, including non small cell lung cancer, pancreatic cancer, bladder cancer and breast cancer. Gemcitabine is a prodrug which is activated intracellularly by phosphorylation into gemcitabine diphosphate and triphosphate. Anti-tumour effects are mediated by a number of mechanisms, including inhibition of ribonucleoside reductase, resulting in depletion of deoxynucleotide pools necessary for DNA synthesis, and incorporation of gemcitabine triphosphate into DNA, resulting in DNA strand termination (Mini, Nobili et al. 2006). Gemcitabine is eliminated through deamination into a uracil metabolite, which is excreted in the urine. The half life of gemcitabine in mice is approximately 30 minutes in the plasma and up to 3 hours in tissues (Shipley, Brown et al. 1992).

Currently, pemetrexed is used as first line treatment in conjunction with cisplatin in patients with mesothelioma (Vogelzang, Rusthoven et al. 2003). The reasons gemcitabine is used instead of pemetrexed as the chemotherapeutic agent in this model are as follows: 1) Pemetrexed is a multi-targeted folate antagonist which inhibits enzymes involved in purine and pyridamine synthesis. In mice, plasma levels of folate and thymidine are around 10 fold higher than humans, meaning the activity of pemetrexed against mouse tumours is limited (van der Wilt, Backus et al. 2001); 2) The immuno-modulatory effects of gemcitabine have been well characterised and gemcitabine is known to synergise with immunotherapy (Nowak, Robinson et al. 2003), whereas the effects of pemetrexed on the immune system are less well known;
3) Since the clinical use of gemcitabine is much greater than that of pemetrexed, results are potentially translatable to more patients with a wider variety of tumour types.
1.8 Aims and hypotheses

Based on an understanding of anti-tumour immune responses as outlined above, I made the following hypotheses:

- The actions of CD4 T cells play a crucial role in orchestrating CD8 and other anti-tumour immune responses and the balance between effector and suppressive CD4 subsets determines whether the immune system rejects or is tolerised to a tumour.

- CD4 T cells can be polarised in vitro into Th1, Th2, Th17 and Treg subsets. Adoptive transfer of *in vitro* differentiated tumour specific effector CD4 subsets will allow us to determine the optimal CD4 T cell anti-tumour response to mediate tumour rejection.

- The immunomodulating effects of cytotoxic chemotherapy mean that the post-chemotherapy environment presents an optimal window for adoptive transfer of anti-tumour effector cells.

In order to investigate these hypotheses I had to establish a model in which the effects of different tumour specific CD4 subsets on anti-tumour immune responses could be studied. Thus the aims of this project were:

- To establish protocols for *in vitro* differentiations of tumour specific Th1s, Th2s, Th17s, Tregs.

- To investigate the activity of *in vitro* differentiated tumour antigen specific CD4 cells against tumours *in vivo* in conjunction with cytotoxic chemotherapy.
• To investigate the effects of cognate help provided by HA specific CD4 subsets on HA specific CD8 T cells during anti-tumour immune responses
2 Materials and Methods
2.1 Tumour cell culture

Cell lines were maintained in RPMI 1640 (invitogen) supplemented with 10% fetal calf serum, 20mM HEPES, 0.05mM 2-mercaptoethanol, 100U/ml penicillin, 50ug/ml gentamicin and Glutamax (Gibco) (R10: complete medium +10% FCS). In addition the neomycin analogue geneticin was added at a concentration of 400ug/ml to all AB1-HA cell lines in order to suppress growth of non-HA expressing cells. Cells were cultured at 37C in the presence of 5% CO2. Cells were passaged when between 80-95% confluent.

Cell passage was performed by washing the adherent monolayer of cells with PBS before incubating them with trypsin at 37C for 1 minute. They were then resuspended in 10ml of complete media, centrifuged, resuspended in complete media and then a proportion of the cell suspension was added to new culture flasks.

For long term storage cells were cooled and resuspended in cold media (R10) supplemented with an additional 20% FCS and 10% DMSO. Cell suspension was aliquoted into cryovials and frozen at -80C for 24 hours. Vials were then transferred for long term storage in liquid nitrogen.

When reviving cells from liquid nitrogen they were gently rewarmed until just above melting point and then transferred into 10ml cold R10, centrifuged, resuspended in R10 supplemented with 10% FCS and transferred to tissue culture vial. After 24 hours media was replaced with R10 containing 400ug/ml geneticin.
2.2 Mice

Balb/c (H-2d) mice were purchased from the Animal Resources Centre (Perth, Western Australia) and housed in the University Department of Medicine.

Transgenic mice (HNT and CL4, both Thy1.2+ve) were purchased from the ARC, Perth. HNT-Thy1.1+ve and CL4-Thy1.1+ve mice, which had been generated by backcrossing onto a Thy1.1 background, were purchased from ARC, Perth. Transgenic mice had been typed individually prior to use by staining peripheral blood samples with anti CD8 and anti VB8.1 for CL4 mice and for HNT mice by staining with anti-CD4 and anti-VB8. Thy1.1 and Thy1.2 homozygotes were identified by staining with anti Thy1.1 and Thy1.2 respectively.

2.3 Tumour inoculations

Tumour cells in an exponential phase of growth were trypsinised at between 80-95% confluence, washed three times with cold PBS and cells were counted. Cells were resuspended in cold PBS at the appropriate concentration and between 5x10^5 and 1x10^6 cells in 100ul were inoculated subcutaneously in the right hind flank.

2.4 Monitoring of mice

Once tumours became palpable they were measured using electronic callipers 3 times per week and then daily when they neared full size. Tumour area was calculated as the product of the two largest, perpendicular diameters. Mice were culled when
tumour area reached 100mm² or if significant ulceration developed. Mice were also culled if their body weight fell below 10% of its starting value or if they developed appearances of looking distressed such as disinterest, hunching or ruffled coat.

2.5 Gemcitabine treatment

Gemcitabine was made up in normal saline by the pharmacy department, Sir Charles Gairdner Hospital, Perth at concentrations of 240mg/ml or 480mg/ml. Gemcitabine was injected intraperitoneally at a dose of 120ug/g or 240ug/g (equal to a volume of 100ul for a 20g mouse).

2.6 Anti-CD40 treatment

Agonist Anti-Cd40 antibody (FGK-45) was purchased from Western Australia Institute of Medical Research (WAIMR) and 100ug of antibody was injected i.p. in 100ul of PBS. Standard treatment regimen was of 3 doses administered every 2 days.

2.7 CD4 depletions

Depleting anti-CD4 antibody (GK 1.5) was purchased from the Western Australia Institute of Medical Research and doses were administered in 100ul of PBS. Injections were performed intravenously for the first dose and if subsequent doses were required these were given i.p. every 3 days for the duration of treatment. CD4 depletion was confirmed by FACS analysis of peripheral blood.
2.8 Preparing single cell suspensions

2.8.1 Spleens and lymph nodes

Organs were harvested into PBS containing 2% FCS and kept on ice. Single cell suspensions were obtained by disaggregation of tissue by gentle mashing between frosted glass slides. Where required, red blood cells in spleens were lysed by resuspending the sample in 2ml of BD Pharm Lyse solution for 2 minutes, followed by addition of 2ml FCS and 5 ml 2% FCS and then centrifugation and resuspension. Suspensions were then strained through 45uM mesh to remove debris before use.

2.8.2 Tumours and other tissues

Whole tissue was harvested into PBS containing 2% FCS and kept on ice. Tissue was injected with 100ul of tumour digest solution (RPMI with 2% FCS(R2) containing 1mg/ml collagenase (LS004176 Worthington Biochemical corporation), 0.1mg/ml DNase I (Roche Applied Sciences)). Tissue was then finely minced using a scalpel and fragments were transferred to a 15ml tube with 1-2ml of tumour digest solution. Samples were digested for 1 hour on rollers at room temperature. 50ul of 0.1M EDTA was added per sample to break up DC-T cell rosettes and samples were left on rollers for a further 15 minutes. Fragments were then allowed to settle and supernatant was strained and transferred into a fresh tube. Samples were then underlayed with 1ml of cold FCS containing 0.01M EDTA and cells were recovered by centrifugation and resuspended in PBS containing 2% FCS or media as appropriate.
2.8.3 Bleeds

Where blood was required from culled mice, cardiac puncture was performed immediately after cervical dislocation. Otherwise peripheral blood was obtained from tail veins by warming the mouse under a heat lamp and making a small nick in the vein with a scalpel. Approximately 50-100ul of blood per mouse was collected into tubes containing 10ul of 1:1000 heparin to prevent coagulation.

2.9 Restimulation of cells for determining cytokine expression

Single cells suspensions were plated into 96 well plates and resuspended in complete media. In some experiments cells were restimulated with PMA, Ionomycin in the presence of Brefeldin A (Leukocyte activation cocktail, BD at 2µl/ml). In some experiments cells were restimulated with PMA 100ng/ml (Sigma-Aldrich P8139) and Ionomycin 2µM (Sigma Aldrich I3909) and Brefeldin A 10µg/ml (Sigma Aldrich B7651) was added after 1 hour. Cells were stimulated for 5 hours at 37°C.

Restimulation of cells with peptide was performed on HNT-thy1.1 +ve in vitro differentiated cells. 5x10^5 splenocytes from a naive thy1.2+ balbc mouse were plated into a round bottom 96 well plate to provide antigen presenting cells. These were cultured overnight in complete media with HNT peptide at 5ug/ml. The following day wells were washed in media and 1x10^5 Thy1.1 HNT cells added to wells. Brefeldin A 10µg/ml was added after 1 hour and wells were harvested after 5 hours. Control wells were treated in the same way but were not pulsed with HNT peptide.
2.10 Cell staining

For surface staining, single cell suspensions were plated into round bottom 96 well plates. Cells were resuspended in 20ul of FACS buffer (PBS containing 2% FCS, 1% bovine serum albumin and 0.01% Sodium Azide) containing 1:200 anti CD16/32 (Trustain, Biolegend) for 10 minutes at 4C, in order to reduce non specific binding. Cells were then resuspended in 20ul of FACS buffer containing fluorochrome labelled antibodies and kept in the dark at 4C for 30 minutes. Cells were washed twice in FACS buffer and resuspended in fixative (BD stabilizing solution) prior to analysis.

For intracellular staining, following surface staining of cells where necessary, cells were plated into 96 well plates, washed and resuspended in fixative (BD FACS lysing solution) for 15 minutes at room temperature. Cells were washed once and if necessary were left overnight at 4C in FACS buffer at this point. Cells were then permeabilized by resuspension in permeabilisation buffer (ebiosciences permeabilisation buffer) for 10 minutes. Cells were then resuspended in 20ul of permeabilisation buffer containing fluorochrome labelled antibodies and left in the dark at 4C for 30 minutes. Cells were washed once in permeabilisation buffer and twice in FACS buffer before being resuspended in fixative prior to analysis. Where CD3, CD4 or CD8 staining was required alongside other intracellular markers, these were all stained together after permeabilisation. Since cells downregulate surface CD4 and CD8 expression during stimulation (Kemp and Bruunsgaard 2001), I found that following restimulation, intracellular staining of these markers gave better delineation of the positive population. Likewise I found that thy1.1/1.2 staining could be performed intracellularly and gave a brighter signal than surface staining. Other than
this, where other surface markers were stained alongside intracellular markers, surface staining was performed prior to fixation and permeabilisation.

### 2.11 Flow cytometry

All flow cytometry was performed on a FACS Canto (Becton Dickinson) except when blood samples were analysed for cell counts, which was performed on a Guava Easycyte Flow Cytometer (see section 2.13). Data was analysed using Flowjo software (Tree star). Compensation was performed using compensation beads (BD Comp beads).

Fluorescence minus one stains (FMOs) were used as controls to set gates. Control wells containing spleen, lymph node or tumour cells were stained with a cocktail of antibodies where one of the antibodies in the panel was omitted and substituted with the relevant isotype control. Thus gates for each antibody could then be set against the FMO for that marker. Examples of gating strategies are shown in the figures where relevant.

To ensure that lymphocyte gates set on forward and side scatter included all lymphocytes of interest, including blasted cells, the population of cells being analysed (e.g CD4+, Thy1.1+ve) was selected from the total population and plotted on forward and side scatter plots. The lymphocyte gate was then applied to this population and could then be adjusted to ensure it included all of the cells of interest.
2.12 Live cell counts

Number and viability of cells in single cell suspensions was determined by staining cells with trypan blue and counting with either a manual hemocytometer, or an automated cell counter (Countess, Invitrogen).

2.13 Cell counts on blood samples and solid organs

In order to perform cell counts on peripheral blood samples, 20μl blood was stained with antibodies to CD3, CD4 and CD8. The volume was then made up to 200μl with BD FACS lysis solution and samples were immediately analysed on a Guava Easycyte flow cytometer. Blood cell counts per ml were automatically generated by the Guava software.

In order to perform cell counts on spleens and lymph nodes, single cells suspensions were prepared from organs as described in section 2.8. Care was taken to rinse all slides and dishes thoroughly during preparation to minimise loss of cells. The mashed organ was resuspended in a fixed volume (V ml) and 200μl was pipetted into a 96 well plate for staining. Thus each sample contained a known proportion of each solid organ = 0.2/V.

Following staining, samples were then resuspended in 200μl and run on the flow cytometer (FACS Canto). In order to calculate what volume of each sample had been analysed it was necessary to know the flow rate of the flow cytometer. The exact flow rate of the flow cytometer was determined by resuspending a known number of beads (BD Trucount Tubes) in 500μl of buffer and running this for 3 minutes. The flow rate at
each setting could therefore be calculated as [(number of beads processed/total number beads) X 500ul / 3]. To compensate for any day to day variation, flow rates were determined at the start and end of each analysis. Day to day variability in flow rates was found to be less than 10% and was within the manufacturers reported values. Samples were then run on the flow cytometer for a fixed period of time. By multiplying the length of time that samples were run by the flow rate of the flow cytometer, the volume of each sample analysed could be calculated. The number of cells in each organ could then be determined by dividing the number of cells counted in the sample by the proportion of each sample analysed and dividing this by the proportion of each organ per sample. Thus the cell count per organ could be calculated as

\[
\text{Cells per 200ul sample (A) = (Cell count) X (0.2ml/volume analysed)}
\]

\[
\text{Total cells in organ = A X (V ml / 0.2 ml)}
\]

Where V is the original volume into which the organ was resuspended.

Since it was not possible to accurately compare absolute cell counts between tumour samples due to inconsistencies in the recovery of cells from digested tissue, an alternative method was needed to determine the concentration of tumour infiltrating lymphocytes such that the frequency of tumour infiltrating lymphocytes could be compared between different tumour samples. This was done by counting the total number of tumour cells analysed per sample from forward and side scatter gates. The ‘total cells’ gate was set to include all cellular events but excluded events lying on the x and y axis, likely to represent debris. To enable comparisons between samples this
gate was fixed across all samples in an experiment. The frequency of tumour infiltrating cells of interest could thus be expressed as a proportion of total tumour cells. Examples of this gating strategy are shown in Figure 5-7A.

2.14 Naive CD4 T cell isolations

Spleens and lymph nodes were harvested from donor mice and single cell suspensions were prepared. Cells were kept on ice throughout in PBS containing 0.5% bovine serum albumin and 2mM EDTA. Cells were counted and CD62L+CD4+ T cells were purified using magnetic columns and a naive T cell isolation kit (Miltenyi Biotec no. 130-093-227) as per manufacturers instructions: Briefly, total CD4 T cells were isolated by negative selection by incubating cells with biotin labelled antibodies against CD8a, CD45R, CD11b, CD25, CD49b, TCRg/d and Ter-119 followed by incubation with antibiotin microbeads. Cells were then passed through a magnetic column and eluted cells collected. In most experiments, in order to improve purity, cells were passed through a second magnetic column at this stage. The elute obtained was then enriched by positive selection for CD62L+CD4+ cells by incubation with CD62L+ microbeads and passage through magnetic column. The column was removed from the magnetic field and cells obtained by flushing the column three times with buffer. The final product typically contained between 83-95% CD4 cells if one negative selection was performed and over 95% CD4 cells if two negative selections were performed. These CD4 cells were 85-95% CD62L+ and contained between 0 - 0.5% CD25+ or CD8+ cells.
2.15 In vitro CD4 T cell differentiations

CD4+CD62L+ CD4 T cells were cultured in 48 well plates in RPMI 1640 supplemented with 10% fetal calf serum, 20mM HEPES, 0.05mM 2-mercaptoethanol, 100U/ml penicillin, 50ug/ml gentamicin, 1X non-essential amino acids (MEM-NEAA, Gibco) and 1X Glutamax (Gibco).

Cells were activated with plate bound anti-CD3 antibody (145.2C11, WAIMR, Perth) and soluble anti-CD28. To precoat wells with anti-CD3, 500ul of PBS containing 5ug/ml anti-CD3 was incubated in wells at 4C overnight. Before the addition of cells, this was aspirated and wells washed once in PBS taking care not to let the wells dry out. Soluble anti-CD28 (BD Pharminogen) at 1ug/ml was then added to culture media where activation was required.

Other cytokines were added to culture media in order to generate different CD4 subsets. These were rmIL-12 (ebioscience 14-8121), rmIL-4 (ebioscience 14-8041), rmIL-6 (ebioscience 14-8061), rh TGFb (Peprotech 100-21), rm IL-23 (R+D systems 1887-ML), rm IL-7 (R+D systems 407-ML), mIL-2 (Roche 11271164001). Neutralising antibodies to IFNg (BD Pharminogen), IL-4 (BD Pharminogen) and IL-12 (BD Pharminogen) were added to cultures where stated. Stimulating anti-ICOS antibody (Biolegend, clone 398.4A) was used in one experiment.

Cells were split at a ratio of 1:2 or 1:3 on day 3 depending on the degree of proliferation. To do this, cells were transferred into conical tubes on ice and wells were rinsed with media which was added to the sample. Cells were centrifuged and washed once with cold media before being resuspended in fresh media containing the appropriate cytokine/antibody cocktail and were plated into fresh wells. Cells were
cultured for 5 or 6 days in total. If media went yellow in between these times, approximately 50% of the media was aspirated, taking care not disturb the cells at the bottom of the well, and replaced with fresh media containing cytokines/antibodies. At the end of the culture period cells were transferred into conical tubes and cell counts and viability were determined by tryphan blue staining and counting using an automated cell counter (Countess, Invitrogen). Cells for in vitro restimulation and staining were washed once with media before use, whereas cells for adoptive transfer into mice were washed three times with cold PBS prior to transfer.

2.16 Adoptive cell transfers

In vitro differentiated cells were washed three times with cold PBS, counted and resuspended in PBS at between $5 \times 10^5$ to $1 \times 10^7$ cells per ml. 100ul ($5 \times 10^5$ – $1 \times 10^6$ cells) were injected iv.

Lymphocytes from CL4 mice were obtained by harvesting axillary, inguinal, popliteal, cervical and mesenteric lymph nodes. Single cells suspensions were prepared and cells were washed once in cold media and twice in cold PBS. Cells were resuspended at $1 \times 10^7$/ml and 100ul ($1 \times 10^6$) was injected iv. Where in vitro differentiated cells and CL4 lymphocytes were co-transferred, cells were resuspended at double these concentrations and mixed at a ratio of 1:1 prior to transfer such that the final injection volume remained 100ul.

For iv injections, mice were warmed under a heat lamp and gently restrained and 100µl was injected iv into a tail vein.
2.17 Statistical analysis

Statistical analysis was performed using Graphpad Prism software. Students t test was used to compare means between groups. Kaplan Meier survival curves were analysed using a Logrank test. Growth curves were analysed using a 2 way ANOVA with repeated measures. Statistical significance was determined at the level of p<0.05, p<0.01 and p<0.001 and these values are reported.
3 How gemcitabine chemotherapy affects the anti-tumour T cell response
3.1 Introduction

This chapter examines the interaction between chemotherapy and CD4 and CD8 T cells and is divided into two parts. Initial experiments examined the effects of cytotoxic chemotherapy on CD4 and CD8 T cell populations. Following this I looked to see how the presence or absence of CD4 cells influenced outcomes to combined treatment with chemotherapy and anti-CD40 directed immunotherapy.

Gemcitabine induces a number of immunomodulatory effects, including lymphopenia, increased antigen cross presentation and cross priming, impairment of humoral immunity (Nowak, Lake et al. 2006), depletion of myeloid derived suppressor cells (Suzuki, Kapoor et al. 2005) and depletion of regulatory T cells (Rettig, Seidenberg et al. 2010). However, the effects of gemcitabine on other CD4 T cell subsets have not been well characterised. My initial experiments suggested the possibility of immune mediated tumour regression after a single dose of gemcitabine. I therefore examined whether the beneficial effects of gemcitabine on anti-tumour immunity were reflected in an altered balance between different CD4 subsets. CD4 subsets were identified by intracellular flow cytometry. Th1s, Th2s and Th17s were defined by functional expression of IFNg, IL-4 and IL-17 respectively, following ex vivo restimulation. Tregs were defined by expression of the transcription factor foxp3, which is a highly specific marker for Tregs in mice (Fontenot, Rasmussen et al. 2005). Since ex vivo restimulation alters the expression of many markers of activation, analysis of some phenotypic markers was restricted to foxp3+ (Treg) and foxp3- (nonTreg) subsets only.

Having examined the effects of gemcitabine on CD4 cells I looked at the role CD4 cells play in mediating outcomes to gemcitabine combined with an immunotherapy.
Previous work using the AB1HA model has shown that gemcitabine synergises with agonist anti-CD40 antibody to cure a high proportion of mice with established tumours (Nowak, Robinson et al. 2003). I examined whether CD4 cells were having an overall positive or negative effect on outcomes to treatment by removing total CD4 cells in vivo using a CD4 depleting antibody. Previous work showed that depletion of CD4s did not affect outcomes to gemcitabine and anti-CD40, suggesting that anti-CD40 substitutes for CD4 help (Nowak, Robinson et al. 2003). However, CD4 cells can help CD8 T cells at points downstream of the priming phase, such as through recruitment and expansion of CD8s at the tumour site (Marzo, Kinnear et al. 2000; Bos and Sherman 2010). I therefore looked to see what the effects of removing CD4 cells were on outcomes to this treatment.

In addition, work in other models has shown that the CD4 response is polarised in an effector or suppressive direction during the early stages of tumour emergence (Darrasse-Jeze, Bergot et al. 2009). I hypothesised that induction of an effective CD8 T cell response may be dependent on the actions of CD4 cells during the early stages of tumour emergence. I therefore looked to see whether removing CD4 cells prior to tumour inoculation affected the outcome to subsequent chemo-immunotherapy.
3.2 Results

3.2.1 Gemcitabine causes transient lymphodepletion and can lead to regression of tumours

Mice bearing 9 day old AB1HA tumours were treated with a single dose of gemcitabine. Analysis of serial blood samples by FACS showed that numbers of CD4 and CD8 T cells declined to around one third of baseline levels after 24 hours but had returned almost back to baseline 4 days later, indicating that gemcitabine causes lymphodepletion but that T cell numbers are rapidly restored within a few days (Figure 3-1A).

Untreated, AB1-HA tumours take between 17 and 23 days to grow to $100\text{mm}^2$. When tumour bearing mice were treated with a single dose of gemcitabine, tumour regression and long term survival was seen in a small proportion (2 of 13) of mice. When growth curves were further analysed it was clear that tumour growth was being affected in two distinct phases (Figure 3-1B). In all mice, within 3 days of administration, tumours regressed to a barely palpable size but then started to regrow at similar rates to that seen pre-treatment. However secondary regression of tumour was seen in some mice between 7 and 12 days after gemcitabine administration. In these mice, tumours rapidly regressed at this point from sizes of up to $15\text{mm}^2$. Thus, in addition to immediate cytotoxicity, gemcitabine can cause a later anti-tumour effect and it is this secondary response which results in complete tumour eradication in a small proportion of mice. This observation was suggestive of immunologically mediated tumour eradication following a single dose of gemcitabine.
Figure 1.1
Counts

Figure B

Harvests

C

Day 0 9 16

Gp 1. Pretreatment
Gp 2. Untreated
Gp 3. Gemcitabine

Day 0

Gemcitabine day 9

Tumour size at harvest

D

Counts

E

Counts

Tumour infiltrating CD4s
pretreatment untreated Gemcitabine

Tumour infiltrating CD8s
pretreatment untreated Gemcitabine
Figure 3-1  Effects of gemcitabine on T cell populations

A  Lymphodepletion in peripheral blood following single dose of gemcitabine. Tail bleeds were performed on mice before, 1 day after and 4 days after 240ug/g gemcitabine i.p (n=5). Cells were stained with antibodies to CD3, CD4, CD8 and cell counts performed using flow cytometry. B Tumour growth curves of individual mice inoculated with AB1-HA on day 0 and treated with 240ug/g gemcitabine i.p on day 9. Shows two independent experiments. C Schedule for harvests of mice treated with gemcitabine (n=8) and pretreatment (n=4) and untreated controls (n=4) and tumour size at harvest for each group. D CD4 and CD8 cell counts on DLN and spleen for groups described in C. E Tumour infiltrating CD3+CD4 and CD3+CD8 T cells expressed as a percentage of total cells, as determined from forward and side scatter plots as shown.

3.2.2  Treatment with gemcitabine results in increased numbers of tumour

DLN CD4 T cells and CD8 tumour infiltration 7 days after treatment

To examine whether the late response to gemcitabine was reflected in changes in T cell populations, I examined T cell populations in lymphoid organs and in tumours 7 days after gemcitabine administration. This timepoint was chosen as this was just prior to the point tumours started regressing, when immune responses were most likely to be observed but when tumours were still of sufficient size to harvest. Mice were treated with a single dose of gemcitabine at day 9 (Figure 3-1C). Organs were harvested at day 16 and analysed by FACS. In order to control for both tumour size and duration of disease, harvests were also performed in a pre-treatment (size matched)control group, and in an untreated (time matched) control group at day 16,
when tumour sizes were larger than in the gemcitabine treated group (Figure 3-1C).

Because tumours in the pre-treatment and gemcitabine treated groups were smaller than the untreated groups, in these groups tumours from 2 mice were pooled prior to staining in order to obtain enough cells for analysis. Thus the data for tumour harvests consists of 2 data points from 4 mice in the pre-treatment group, 4 data points from a total of 8 mice in the gemcitabine treated group and 4 data points from 4 mice in the untreated group.

The number of total CD4 T cells, was found to be increased in the tDLN of gemcitabine treated mice (Figure 3-1D) 7 days after treatment, compared with untreated mice. Tumour sizes were larger in the untreated group, so the increase in CD4 T cells in the DLN of gemcitabine treated mice was seen despite an overall reduced tumour load. Cell counts were not available for the pretreatment group. There was no change in the numbers of splenic CD4s or CD8s. When tumour infiltrating T cells were examined, the frequency of CD4 cells within the tumour was not increased by gemcitabine. In contrast, there was a large increase in the population of tumour infiltrating CD8 cells compared to both the pre-treatment and untreated controls (Figure 3-1E).

3.2.3 Gemcitabine leads to a reduction in tumour infiltrating T regs and an increase in tumour infiltrating Th2

To assess the effects of gemcitabine on T regs, samples were stained intracellularly for foxp3 expression (figure 3.2A). Mice treated with gemcitabine had a reduced proportion of foxp3+ CD4 T cells in the DLN after 7 days compared with that seen pre-treatment (figure 3.2B). However there was no difference in proportions of foxp3+
cells between the untreated and treated group at day 16, so the decline in foxp3+ cells in the DLN could have been a feature of prolonged disease duration rather than gemcitabine treatment. In contrast, gemcitabine lead to a substantial reduction of foxp3+ CD4 T cells in the tumour at day 7 compared to both the untreated and the pre-treatment group (figure 3.2B), demonstrating that this effect was not confounded by differing tumour sizes or disease duration. In addition, gemcitabine had no effect on the proportion of foxp3+ CD4 cells in non tumour draining lymph nodes or the spleen, indicating that the reduction in Treg frequency was not a systemic effect but was localised to the tumour microenvironment.

To assess whether the reduced frequency of Tregs seen post gemcitabine could have been due to alterations in proliferation of regulatory or effector subsets, cells were stained intracellularly for Ki67, a marker expressed by proliferating cells. Overall, Ki67 was expressed by a greater proportion of Tregs than non-Tregs (figure 3.2C) and demonstrated high rates of proliferation amongst intratumoural Tregs.

In the tumour, gemcitabine led to a small but significant reduction of Ki67+ Treg cells compared to untreated animals (p<0.01) and a non-significant reduction when compared to the pretreatment group (p=0.08). In contrast, the opposite effect was seen on the foxp3- (nonTreg) CD4 cells; during tumour growth the frequency of Ki67+ foxp3- CD4 cells within the tumour declines between the pretreatment and untreated group (p<0.01). However treatment with gemcitabine reversed this decline, significantly increasing the frequency of Ki67+ foxp3- CD4 cells compared to untreated mice (p<0.01).
Figure 3-2  Effect of gemcitabine on T reg and non-Treg CD4+ cells

A  Gating on foxp3+ and foxp3- CD4 cells and on CD4+foxp3+Ki67+ cells.  B  Proportion of foxp3+ CD4 cells in DLN, NDLN, spleen and tumour in pretreatment, untreated and gemcitabine treated groups described in figure 1.1C.  C  Ki67 expression on foxp3+ and foxp3- CD4 cells.
Although these differences were small, these results suggest that the reduction in the proportion of tumour infiltrating Tregs seen 7 days after gemcitabine treatment may in part be due to localised changes in the tumour microenvironment, resulting in both reduced proliferation of Tregs and increased proliferation of non-Treg CD4 T cells compared to untreated mice.

To see whether gemcitabine causes any concomitant increases in CD4 Th1, Th2 or Th17 helper subsets, samples were restimulated ex vivo with PMA and ionomycin in the presence of brefeldin A and then stained intracellularly for IFNg, IL-4 and IL-17 cytokine expression (Figure 3-3). This showed that IFNg expression by CD4 cells declined in the spleen between days 9 and 16 of tumour growth (pretreatment vs untreated controls, p<0.01). Treatment with gemcitabine had no effect on IFNg expression compared with untreated controls. Similarly, frequencies of IL-4 producing CD4 cells, but not IL-17+ CD4+ cells, declined between pre-treatment and untreated groups in the DLN and spleen, although overall the frequency of these subtypes was low, at less than 1% of all CD4 T cells. The tumour contained greater proportions of IFNg+, IL4+, and IL-17+ CD4 cells than were seen in either the spleen or the DLN at any timepoint. Within the tumour, the frequency of IL-4 secreting Th2 cells was increased by gemcitabine, with no change in the frequency of Th1s or Th17s.

These results indicate that there is a decline in tumour infiltrating Tregs 7 days after gemcitabine which is partially counterbalanced by an increase in tumour infiltrating Th2s.
Figure 3-3  Effects of gemcitabine on effector CD4 subsets

Mice were treated with 240ug/g gemcitabine and harvests were performed as per schedule described in figure 1.1c. Cells were restimulated ex vivo and expression of IFNg, IL-4 and IL-17 determined through intracellular flow cytometry. A Gating on IFNg+, IL4+ and IL-17+ CD4 cells. B Expression of IFNg, IL-4 and IL-17 on CD4 cells recovered from DLN, spleen and tumour.
3.2.4 The increase in tumour infiltrating CD8 T cells following gemcitabine treatment is not associated with increased intratumoural CD8 proliferation

CD8 T cells were examined in the same experiment at the time points indicated in Figure 3-1A. In the draining lymph node and spleen 7 days after treatment, gemcitabine did not change the numbers of CD8 T cells when compared to untreated mice (Figure 3-1D). However there was a significant increase in tumour infiltrating CD8 T cells in comparison to pre-treatment and untreated controls (Figure 3-4A). These cells were stained for Ki67 to determine whether this could be due to increased proliferation (Figure 3-4B). Surprisingly, despite being present in greater quantities, the frequency of Ki67 expression on tumour infiltrating CD8 T cells from gemcitabine treated tumours was substantially less than that seen pre-treatment or in untreated tumours (Figure 3-4C). This indicates that the accumulation of CD8 T cells in these tumours is unlikely to be due to increased local proliferation.

The functional capacity of tumour infiltrating CD8 T cells was examined by restimulating cells ex vivo with PMA and ionomycin and staining for intracellular IFNg. There was no significant difference in the percentage of CD8 cells which produced IFNg between any of the groups. In addition the proportion of IFNg producing CD8s expressed as a percentage of total tumour cells was no different between the groups (Figure 3-4D), showing that although gemcitabine causes increased CD8 accumulation within the tumour, the number of IFNg secreting CD8s did not increase.
Figure 3-4   Effects of gemcitabine on CD8 T cells

Mice were treated with 240ug/g gemcitabine and harvests were performed as per schedule described in figure 3.1c and gating performed as shown in figure 3.1E.  A Tumour infiltrating CD8 T cells expressed as a percentage of total cells.  B Gating of CD8+Ki67+ cells.  C Ki67 expression on CD8s recovered from DLN, spleen and tumour.  D Proportion of IFNg+ CD8 cells, analysed by restimulation ex vivo and intracellular flow cytometry – shown as a proportion of all CD8 cells and as a proportion of total tumour cells.
3.2.5 CD4 T cell depletion changes the efficacy of combination gemcitabine and agonist anti-CD40 treatment

Although the above results suggest that gemcitabine alters the balance between effector and suppressive CD4 subsets within the tumour microenvironment and promotes CD8 infiltration of the tumour 7 days after administration, this is not sufficient to cure mice in the majority of cases. I next investigated the role CD4 cells play during combination chemo-immunotherapy with gemcitabine and agonist anti-CD40 antibody. This combination was chosen as it has been previously shown to cure between 60-80% of mice with established tumours and provides one of the highest cure rates associated with this model. I hypothesised that depleting CD4 cells would have a negative effect on outcomes to this treatment.

We treated tumour bearing mice according to previously established protocols, with 5 doses of gemcitabine followed by 3 doses of agonist anti-CD40 antibody. CD4 cells were depleted in vivo by injection with GK1.5 antibody during two phases: an early phase from before tumour inoculation to the commencement of treatment and a late phase from the commencement to the end of treatment (Figure 3-5A). By depleting CD4 cells I hoped to see what the overall effect of the total CD4 population was during these two phases and thus whether suppressive or effector CD4 subsets were likely to be dominant. CD4 cells were depleted in these two phases in order to ascertain how the CD4 T cell response was polarised during the early stages of tumour emergence and whether this differed to what was seen during treatment with chemo-
The effects of CD4 depletion on outcomes to treatment with gemcitabine and agonist anti-CD40

Figure 3-5
A Treatment schedule. Mice were inoculated with AB1-HA on day 0 and treated where indicated with 5 doses of gemcitabine 120ug/g i.p. every three days commencing day 9, followed by 3 doses of anti-CD40 (FGK45) 100ug i.p. every two days commencing day 23. CD4s were depleted as indicated in an ‘early’ phase from day -1 to day 5 and a ‘late’ phase from day 8 to day 25, through injection of anti-CD4 antibody (GK1.5) initially at 150ug iv and then 100ug i.p. every 3 days thereafter. B Confirmation of CD4 depletion by flow cytometry. C Survival curves and tumour growth curves of non-surviving mice (n=5 mice per group).

immunotherapy. The efficacy of CD4 depletion was confirmed by FACS analysis of peripheral blood samples (Figure 3-5B).

3.2.6 Anti-CD40 delays tumour growth compared to gemcitabine treatment alone

Following commencement of treatment with gemcitabine at day 9, tumours rapidly regressed from a size of around 2mm diameter to barely palpable. However, 8 days after completion of gemcitabine, all tumours had started to regrow at a rate similar to that seen in untreated mice and there were no long term survivors (Figure 3-5C). In mice that received anti-CD40 treatment following gemcitabine, tumour regrowth was significantly delayed by a further 10 days compared to those that received gemcitabine alone, although all eventually regrew tumours. One mouse in this group died unexpectedly during treatment with no apparent cause evident on post mortem.
3.2.7 Early CD4 depletion improves survival whereas late CD4 depletion accelerates tumour growth in mice treated with gemcitabine and agonist anti-CD40 antibody

In mice treated with gemcitabine and anti-CD40, depletion of CD4 cells prior to inoculation, but ceasing before commencement of treatment (early depletion) was associated with a small but significant improvement in survival (Figure 3-5C), indicating an overall suppressive effect of the CD4 population during this phase. 1 out of 5 mice in this group survived long term. In contrast, CD4 depletion during treatment (late depletion) did not have any effect on survival, when compared with those treated with gemcitabine and anti CD40 alone, with 1 out of 5 mice remaining tumour free at the end of the study period. However in those mice that did regrow tumour, late CD4 depletion significantly hastened time to tumour regrowth compared with undepleted or early depleted groups (Figure 3-5C).

3.2.8 Following early depletion, regenerating CD4 T cells are highly activated and contain increased frequencies of both Th1s and Tregs.

In order to examine how T cell populations were affected by gemcitabine and anti-CD40 and the effects of removing CD4 cells on CD8 T cells, we harvested tumour draining lymph nodes, non-tumour draining lymph nodes and spleens from mice at day 0, day 8 (pre-treatment) and day 28 (post treatment) and analysed T cell populations by flow cytometry (}
Figure 3-6 Effects of CD4 depletion on CD4 T cells in mice treated with gemcitabine and anti-CD40

A ICOS expression; B foxp3 expression; C IFNg expression on CD4 cells from mice treated according to schedule in figure 1.5A. Harvests performed before tumour inoculation (day -1), before treatment commenced (day 8) and after treatment completed (day 28) in mice treated with gemcitabine and anti CD40 and depleted of CD4.
CD4 cells where indicated (NB - CD4 cells absent in early depleted mice at day 8 and in late CD4 depleted mice at day 28). 3 mice per group.

The frequency of activated CD4 T cells, as determined by expression of ICOS, showed a progressive increase during tumour emergence and treatment in all compartments (Figure 3-6A). However, in mice that received early CD4 depletion, over 90% of the CD4 cells which had regenerated by day 28 displayed an activated (ICOS+ve) phenotype.

The frequency of foxp3+CD4+ cells within the DLN, NDLN and spleen did not significantly change during treatment with Gemcitabine and anti CD40 (Figure 3-6B). Likewise, the percentage of IFNg-expressing CD4 cells did not alter in the DLN or NDLN but was reduced in the spleen at day 28 (Figure 3-6C). However, in those mice that received early CD4 depletion, the frequency of both foxp3+ and IFNg+ CD4 cells was significantly increased at day 28 in all compartments compared with mice that did not receive CD4 depletion.

3.2.9 CD8 T cells display a more activated phenotype when CD4 T cells are depleted

Between day -1 and day 28, the expression of ICOS on CD8 cells increased significantly in the DLN and spleen, but not in the NDLN, of mice treated with gemcitabine and anti CD40 (Figure 3-7). In mice that received CD4 depletion, a significant increase in the frequency of ICOS+ CD8 cells compared to undepleted mice was observed in the DLN.
and NDLN following both early and late depletion. In addition, the effects of early depletion on the frequency of ICOS+ CD8s persisted at day 28, 23 days after the last dose of depleting antibody. In the spleen, early CD4 depletion increased the frequency of ICOS+ CD8s at day 8, but there was no difference at day 28 between undepleted, early depleted or late depleted groups.

Overall, these results suggest that treatment with gemcitabine and anti-CD40 leads to increased activation of CD8 T cells. Since removal of CD4s increases the frequency of ICOS expression by CD8 T cells, it would appear that CD4 cells are having a suppressive effect at the level of CD8 activation during both the early and the late (treatment) phases.

However, despite the suppressive effect of CD4 T cells on CD8 priming, removal of CD4s during the treatment phase accelerated tumour growth, suggesting that during treatment CD4s are having additional, positive anti-tumour effects unrelated to CD8 activation.
Figure 3-7  Effects of CD4 depletion on CD8 T cells in mice treated with gemcitabine and anti-CD40

ICOS expression on CD8 T cells in DLN, NDLN and spleen of mice treated according to schedule in figure 1.5A. Harvests performed before tumour inoculation (day -1), before treatment commenced (day 8) and after treatment completed (day 28) in mice treated with gemcitabine and anti CD40 and depleted of CD4 cells at timepoints indicated. 3 mice per group.
3.3 Discussion

This chapter examines the interaction between gemcitabine and T cell immunity. It was clear from initial treatment experiments that a single dose of gemcitabine led to tumour regression which was not due to direct cytotoxicity on tumour cells. This occurred after the period of lymphodepletion and homeostatic proliferation, which was shown to last around four days. This suggests that during this initial window, gemcitabine could be priming an immune response, or removing a brake from a pre-existing immune response, which was then able to lead to tumour regression some 7 to 12 days later. This would be consistent with the ability of gemcitabine to cause an immunogenic, apoptotic cell death, increase antigen presentation and cross priming and deplete suppressive cells. To confirm that this response is immunologically mediated this experiment could be repeated in immunodeficient mice. Another way of confirming the ability of gemcitabine to induce immunogenic cell death would be to immunise mice with gemcitabine treated AB1HA cells and then rechallenge mice with tumour after an interval, with tumour rejection indicating the induction of memory responses. This could be compared to the immunity generated by other HA stimuli, such as influenza vaccines, or to responses to tumour cells treated by other chemotherapeutic agents known to induce immunogenic cell death (Tesniere, Schlemmer et al. 2009).

I was interested to see if these responses were reflected by changes in T cell populations. 7 days after gemcitabine administration I found that there was an increase in total CD4 cells in the draining lymph nodes, but not in the spleen, of gemcitabine treated mice. This could be due to increased antigen delivery to the
tumour draining lymph node as a result of gemcitabine induced tumour cell death. In contrast numbers of CD8 T cells were not affected by gemcitabine treatment when compared to untreated controls. In the tumour there was no change in the frequency of tumour infiltrating CD4 cells, however there was a substantial increase in CD8 tumour infiltration following gemcitabine treatment. These CD8s displayed reduced levels of proliferation compared to untreated mice, suggesting that increased trafficking into the tumour and/or improved survival was more likely to explain the increased infiltrate than in situ proliferation.

CD4 cells have been shown to help CD8 infiltration of tumours and to virally infected tissues (Marzo, Kinnear et al. 2000; Nakanishi, Lu et al. 2009). Although the increase in tumour infiltrating CD8 T cells could not be explained by increases in total CD4 infiltration, when the composition of the intratumoural CD4 T cells was examined, a substantial reduction in the proportion of Tregs was observed. This effect was localised to the tumour microenvironment. The reduction in intratumoural Tregs observed following gemcitabine was associated with reduced rates of proliferation within the Treg population and increased proliferation of nonTreg CD4s compared to untreated controls. In addition, in all treatment groups the Treg population contained much higher frequencies of proliferating cells than the non-Treg population, with the highest rates observed within the tumour. Since gemcitabine targets proliferating cells, this disparity could explain how gemcitabine might preferentially deplete Tregs over non-Tregs. This would be similar to the mechanism by which cyclophosphamide selectively depletes Tregs (van der Most, Currie et al. 2009).

Gemcitabine did not increase the frequency of tumour infiltrating Th1s and Th17s, the two CD4 subsets believed to have the most potent anti-tumour activity. Instead, an
increase in tumour infiltrating Th2s was observed. It should be noted however that even after gemcitabine the frequency of intratumoural Th2s remained low, at less than 5% of the total tumour infiltrating CD4s, whereas Th1s still constituted over 15% of the CD4 population.

The effect of gemcitabine on the composition of CD4 subsets in peripheral blood has been examined previously. Rettig et al found that gemcitabine lead to a brief, transient reduction in foxp3+ CD4 cells in peripheral blood of mice and patients after treatment with gemcitabine (Rettig, Seidenberg et al. 2010). In contrast, Plate et al found no effect of gemcitabine on the frequency of circulating CD4+CD25+ cells, which are likely to be highly enriched for Tregs (Plate, Plate et al. 2005). However the effects of gemcitabine on intratumoural CD4 populations have not been characterised. A previous study using the same AB1HA model found that mice treated with 5 doses of gemcitabine had an increased histological total CD4 infiltrate compared with untreated mice (Nowak, Robinson et al. 2003). My findings expand on this and suggest that gemcitabine induces localised changes within the tumour microenvironment resulting in a reduction in the frequency of tumour infiltrating Tregs.

It is not possible to tell from these results whether gemcitabine acts on CD4s or CD8s directly or whether the changes in T cell populations observed result from other, indirect effects. I did not examine tumours for myeloid derived suppressor cells (MDSC), which are known to be depleted by gemcitabine (Suzuki, Kapoor et al. 2005; Sinha, Clements et al. 2007). MDSC have been shown to promote Treg development (Huang, Pan et al. 2006) and decrease macrophage production of type 1 cytokines, including IL-12 (Sinha, Clements et al. 2007), an effect reversed by gemcitabine.
Depletion of MDSCs by gemcitabine could be a mechanism which explains the observed changes in intratumoural T cell subsets.

Although gemcitabine induced many localised changes to the tumour microenvironment which could be considered favourable to anti-tumour immune responses, it is important to note that in the majority of mice this was not sufficient to cure the tumour. There are a number of reasons why this may be the case. The Tregs that remain after gemcitabine treatment may still be able to impair tumour rejection. In this model gemcitabine favoured an increased Th2 response which may not be the most favourable effector CD4 subset. Additionally, although the CD8 infiltrate was increased overall, there was no increase in the numbers of IFNg secreting CD8s and the CD8s showed reduced proliferation in situ, suggesting that they may be functionally impaired (Plate, Plate et al. 2005). Therefore there may still be factors limiting the effectiveness of the local immune response, despite the beneficial effects seen with gemcitabine.

In the second half of this chapter I examined the effects of CD4 cells on outcomes to chemo-immunotherapy. Gemcitabine combined with agonist anti-CD40 mAb improved survival in mice inoculated with the mesothelioma cell line AB1-HA. However, there were no cures with this treatment. Previous published reports using this same model and treatment protocol have achieved cures in up to 80% of mice (Nowak, Robinson et al. 2003). One possible reason for this discrepancy is due to the differences in growth rates of the tumour cells. By day 9 the tumours in this study were slightly larger than those in previous studies, suggesting the line used here is more aggressive.
I found that prophylactic depletion of CD4 cells improved survival in this model. This suggests that regulatory CD4 T cells are dominant over effector CD4 subsets during the period of tumour emergence, leading to overall suppression of anti-tumour responses by the CD4 T cell population. In contrast, CD4 depletion during the treatment phase did not alter overall survival, although in those mice which regrew tumour, time to tumour outgrowth was hastened, suggesting there may be an overall positive CD4 mediated anti-tumour effect during this phase. These results require confirmation with a repeat experiment, which was not possible due to time constraints.

These results are consistent with previous studies which have similarly demonstrated that prophylactic (Onizuka, Tawara et al. 1999; Teng, Swann et al. 2010), but not therapeutic (Quezada, Peggs et al. 2008), T reg depletion improves outcome. Teng, Swann et al compared three different methods of prophylactic T reg depletion – anti CD4 mAb, anti CD25 mAb and anti FR4 mAb - and found equivalent survival benefits, all of which were dependent on CD8 cells. Darasse-Jeze et al specifically examined the kinetics of early effector T cell (Teff) and Treg responses following initial encounter with tumour antigen (Darrasse-Jeze, Bergot et al. 2009). They found that Tregs expanded more rapidly and prevented activation of naive Teffs. If memory Teffs were present, however, Tregs were unable to control Teff expansion and tumours were rejected. This suggests that in naive mice without memory effector T cells, Tregs will be dominant during tumour emergence.

Following early depletion, the CD4 cells which regenerated by day 28 displayed a highly active phenotype with higher proportions of both Foxp3 expressing and IFNγ producing cells. This is consistent with work in humans showing that homeostatic proliferation expands Treg cells, leading to increased proportions of CD4+CD25hi CD4
cells for up to 6 months following chemotherapy (Zhang, Chua et al. 2005). In the early depleted group survival was improved despite the increased percentage of regulatory cells, indicating that the T regs may be ineffective or irrelevant at this later stage of the immune response, or that the positive effects of the observed increased population of IFNg secreting effector T cells outweigh the negative effects from Tregs. Reduced sensitivity of T cells undergoing homeostatic proliferation to inhibitory signals (Shvets, Chakrabarti et al. 2009) could also explain the improved outcomes seen despite the increased frequencies of Tregs.

I examined the effects of CD4 depletion on CD8 activation and found that the frequency of activated CD8s was increased in the absence of CD4 cells at all points examined. This suggests there is a CD4 mediated impairment of CD8 T cell activation, which may explain in part how early CD4 depletion improves anti-tumour activity. However activated CD8s also increased to a similar level at day 28 in the late depleted group, where tumour growth was accelerated by CD4 depletion. It is therefore possible that despite being activated these CD8s still require CD4 help to fulfil effector function. This would be consistent with studies suggesting that CD4 cells are needed in the effector phase as well as the priming phase of the CD8 anti tumour response (Marzo, Kinnear et al. 2000; Nakanishi, Lu et al. 2009; Bos and Sherman 2010).

This experiment shows that the overall tone of the CD4 response is suppressive at the time of initial tumour antigen encounter but may have positive anti-tumour effects during treatment with gemcitabine and anti CD40 mAb. It should be acknowledged that although depletion of CD4s during early and late phases had a small impact on tumour growth, these effects were less than anticipated and did not substantially alter the number of mice cured. As such it cannot be concluded from these results that CD4
T cells are a critical determinant of outcome in this model. This could be because agonist anti-CD40 treatment replicates one of the main mechanisms by which CD4s provide help, masking the effects of CD4 depletion. Or it could be that the opposing actions of effector and suppressive CD4 subsets mean that the net effect of removing total CD4 cells is attenuated. Given that we observed tumour regression 7-12 days after a single dose of gemcitabine it is also possible that using a schedule whereby repeated doses of gemcitabine are given every 3 days may be depleting rather than enhancing the emerging CD4 response, minimising the effect of further CD4 depletion.

The results described in this chapter provided a rationale for experiments described in the following chapters to see if we could take advantage of the immunological changes induced by gemcitabine to further skew the CD4 anti-tumour response in a way that achieved tumour rejection. Since a single dose of gemcitabine was shown to stimulate anti-tumour immunity and due to the possible confounding effects of the combined gemcitabine and anti-CD40 schedule, in all future experiments mice were treated with a single dose of gemcitabine.
Establishing a model to study the anti-tumour effects of CD4 subsets
4.1 Introduction

I hypothesised that adoptive transfer of tumour specific CD4 subsets would promote tumour rejection. This chapter describes the establishment of a model with which this hypothesis could be tested, by generating Th1, Th2, Th17 and Treg subsets in vitro and adoptively transferring them into tumour bearing mice.

The first half of this chapter describes the optimisation of protocols for in vitro differentiation of CD4 subsets. To achieve this, naive T cells require TCR activation with co-stimulation in the presence of the appropriate polarising cytokines. Naive T cells express high levels of CD62L, meaning that they can be isolated from bulk CD4 cells by positive selection using CD62L targeted antibodies. I activated naive CD4 cells with an activating anti-CD3 antibody. CD3 is a transmembrane protein which associates with the T cell receptor to form the TCR complex. Activating CD3 antibodies can mimic TCR activation signals in lymphocytes, leading to cell activation and proliferation (Wacholtz, Patel et al. 1989) and their use is well established in in vitro lymphocyte differentiation protocols (Zhang, Zhang et al. 2003; Harrington, Hatton et al. 2005). Costimulation through CD28 activation is also required to ensure lymphocytes develop into effector cells, the absence of CD28 signalling leading to anergy or deletion (Appleman, Tzachanis et al. 2001).

I then had to determine the optimal combination of cytokines for CD4 subset differentiation protocols. Previous studies have shown that IL-12 is sufficient to induce Th1 differentiation and IFNγ production by cells activated in vitro (Zhang, Zhang et al. 2003) although IFNγ itself also promotes Th1 differentiation (Kaiko, Horvat et al. 2008). IL-4 drives the formation of Th2 cells (Nishimura, Iwakabe et al. 1999), and Th2
differentiation can also be enhanced by IL-2 (Zhu, Cote-Sierra et al. 2003). Th17 differentiation requires TGFβ and IL-6 (Harrington, Hatton et al. 2005; Park, Li et al. 2005). Tregs can be induced from naive precursors through activation in the presence of TGFβ alone (Yang, Nurieva et al. 2008). By activating naive CD4 cells in cultures supplemented with these cytokines I aimed to induce differentiation into each of these subsets.

In order to generate HA specific CD4 subsets I then applied these protocols to naive CD4 cells harvested from HNT mice, which express a T cell receptor that recognises a class II restricted HA epitope. I hypothesised that adoptive transfer of tumour (HA) specific CD4 subsets into mice bearing an HA expressing tumour mice would enable us to study the effects of different CD4 subsets on anti-tumour immunity. Therefore it was important to determine how in vitro differentiated CD4 cells behaved in vivo after adoptive transfer into mice, whether these cells survived, were able to respond to tumour antigen and whether they retained the phenotype imposed on them during in vitro culture.

The second part of this chapter describes experiments characterising the behaviour of in vitro differentiated cells in vivo following adoptive transfer. To do this I made use of the fact that there are a number of different alleles of the thy 1 (CD90) lymphocyte marker in mice and for some of these alleles, thy1.1 and thy1.2, specific antibodies are available. BALB/c HNT mice had been backcrossed onto a thy1.1 balb/c background. Homozygous thy1.1+HNT BALB/c mice were then used as the donor source for adoptively transferred CD4 cells. Recipient mice were thy1.2+ balb/c, thus when mice were culled and recovered cells analysed by flow cytometry, adoptively transferred thy1.1+ cells could be identified from endogenous thy1.2+ cells by staining with an
anti-thy1.1 antibody. This enabled me to track the fate, distribution and activity of the adoptively transferred in vitro differentiated CD4 subsets in vivo.
4.2 Results

4.2.1 Generating in vitro polarised CD4 subsets

Initial experiments were aimed at determining the optimal combination of cytokines and neutralising antibodies to drive the differentiation of naive CD4 T cells into helper T cell and T reg subsets. These subsets were defined by expression of the signature cytokine – IFNg for Th1s, IL-4 for Th2s, IL-17 for Th17s – or by foxp3 expression in T regs, as determined by intracellular flow cytometry.

Naive (CD62L+) CD4 cells were isolated from splenocytes using magnetic beads, through negative selection of CD4 cells followed by positive selection of CD62L+CD4+ cells. A single negative selection of CD4s produced a purity of between 83-92% over a number of different experiments. In order to optimise the purity of naive CD4+ cells for in vitro differentiation, cells were passed twice through the negative selection column, which produced a final population of >95% CD4+ cells which were >90% CD62L+ve with no detectable CD25+ or CD8+ contamination (Figure 4-1A).

All cells were plated in 48 well plates at 3-5x10^5 cells per well and activated with plate bound anti-CD3 and soluble anti-CD28. By day 3 the cells had proliferated to around 1-2x10^6 cells per well and required splitting at a ratio of between 1:2 and 1:3. If media went yellow in between these times, half the media was aspirated and replaced with fresh media containing cytokines.

In initial experiments cells were activated continuously with anti CD3 and antiCD28 for the full 5 or 6 days of culture. However it soon became clear that continuous activation adversely affected the viability of some of the subtypes and so I then tried
removing the activating stimulus at the day 3 split by washing them in media and
plating them in cytokines without additional anti CD3 or anti CD28 for the remainder
of the culture period. Although these problems had to be addressed concurrently, I
will discuss the issues involved in generating CD4 T helper phenotypes and in
maintaining cell viability separately in the next two sections.
A Isolation of naive CD4s from splenocytes. Graphs show CD4, CD25, CD62L, CD8 expression on cells before isolation (green line), after negative selection of CD4+ cells (blue) and after positive selection of CD4+CD62L+ cells (black). Bold line and label indicate final percentages. B FACS plots of cells cultured in Th1, Th2, Th17 and Treg conditions with the addition of IL-2 or neutralising antibodies where indicated. Cells restimulated with PMA and ionomycin prior to intracellular flow cytometry. Gated on CD4+ cells.

Figure 4-1  
In vitro differentiation of CD4 subsets
4.2.2 Generating phenotypes of in vitro differentiated CD4 subsets

Th1s

IL-12 promotes Th1 differentiation by STAT-4 mediated activation of t-bet. In addition, IFNg acts in an autocrine fashion to further promote t-bet expression, however IL-12 alone is sufficient to cause Th1 development in vitro cultures (Zhang, Zhang et al. 2003). I compared different concentrations of IL-12 and found that 5ng/ml was sufficient to induce around 60% IFNg expression in cells activated with anti-CD3 and anti-CD28 for 5-6 days. The addition of neutralising anti-IL-4 antibodies further increased the percentage of cells expressing IFNg (Figure 4-1B).

Th2s

Th2 differentiation is driven by IL-4 but is inhibited by IFNg. When naive CD4 T cells were activated with anti CD3 and anti CD28 in the presence of IL-4 and neutralising anti-IFNg for 5 or 6 days a proportion of cells (between 0.5-50% over a number of different experiments) stained intracellularly for IL-4 (Figure 4-1B). IL4 expression was higher if cells were stimulated for 6 days than if stimulation was removed after day 3, however the viability of the cells deteriorated if the stimulation was continued beyond day 3 (see section 4.2.3).

The addition of IL-2 increased the percentage of IL-4 producing cells when stimulation was removed after 3 days but had no effect on IL-4 expression when cells were stimulated for the full culture period (Figure 4-1B), suggesting that exogenous IL-2 can compensate in part for the loss of CD3 and CD28 stimulation on Th2 differentiation, but is superfluous in the presence of continued activation.
In some experiments a small percentage of cells activated in Th2 culture conditions expressed IFNγ after restimulation. IFNγ antagonises Th2 development and so it was important to remove any factors which may be driving a Th1 rather than a Th2 phenotype. I therefore tried adding neutralising anti-IL-12 antibodies to Th2 cultures, however this did not improve the yield of IL-4 +ve cells (Figure 4-1B).

Studies using splenocytes pulsed with TCR specific peptide to activate CD4 cells in vitro have been able to generate higher levels of intracellular IL-4 than I was able to achieve (Nishimura, Iwakabe et al. 1999). It was possible that antigen presenting cells within these cultures may be delivering signals through receptors other than CD3 and CD28 which may promote Th2 development. ICOS is a costimulatory molecule of the CD28 family that is expressed on activated T cells and stimulation through ICOS has been shown to favour Th2 development (Shilling, Clay et al. 2009). Therefore I tried adding stimulating anti-ICOS antibody to Th2 cultures. However this also had no effect on yield of IL-4 producing cells (Figure 4-1B).

Lack of detection of IL-4 on intracellular staining could be due to inadequate production, or it could be due to inadequate detection methods. I restimulated cells with plate bound anti-CD3 and soluble CD28 in the presence of Brefeldin-A, but levels of IL-4 induced by this were comparable to PMA and ionomycin stimulation. I also stained cells for IL-5 expression in addition to IL-4 but this did not substantially increase the identification of Th2 cells (Figure 4-1B).

In summary, I was only able to detect high frequencies (over 15%) of IL4 producing cells if cells were stimulated continuously for 5 to 6 days, however, as discussed in section 4.2.3, this resulted in unacceptable levels of cell death.
**Th17s**

Th17 cells can be derived from naive precursors through TCR stimulation in the presence of TGFβ and IL-6. I was able to induce between 20-50% of cells to produce IL-17 by stimulation with plate bound anti-CD3 and soluble anti-CD28 for 5 or 6 days in the presence of 2.5ng/ml TGFβ and 20ng/ml IL-6 (Figure 4-1B). IL-23 is not required for Th17 differentiation but has been shown to be an important survival factor for Th17s and was therefore included in culture conditions.

Other factors have also been shown to promote IL-17 expression in CD4 T cells, including the type of media used. Veldhoen et al found that CD4 T cells activated in Th17 polarising conditions in IMDM produced more IL-17 secreting cells than those grown in RPMI and this was due to the presence of AhR ligands in IMDM media (Veldhoen, Hirota et al. 2009). Similarly I found that IL-17 expression was approximately 2 fold higher in Th17 cultures grown in IMDM than RPMI, whereas the choice of media did not make any difference to the other subsets.

**Tregs**

In mice foxp3 is a highly specific marker for the regulatory T cell subset (Fontenot, Rasmussen et al. 2005). Foxp3+ CD4 T cells can be induced from naive precursors by TCR activation in the presence of TGFβ. I found that 10ng/ml of TGFβ in addition to neutralising IFNγ antibodies and CD3/CD28 activation produced between 50-90% foxp3+ cells (Figure 4-1B).

**Th0s**

In order to provide a control for later experiments I also generated CD4 T cells which had been activated in the same way as the other CD4 subsets but had not been
differentiated into specific subsets. This was done by activating naive CD4 T cells with anti CD3 and anti CD28 for three days and then removing the activation and adding IL-2 for the remaining two days. Neutralising anti IFNγ and anti IL-4 antibodies were added to inhibit differentiation into Th1 or Th2 phenotypes. These cells proliferated to a similar extent as the other subtypes and cultures typically contained less than 10% IFNγ+ cells and less than 1% IL-4, IL-17 or foxp3+ cells.

4.2.3 Maintaining cell viability of in vitro differentiated CD4 subsets

It was clear from these early attempts at CD4 differentiation that although it was possible to induce variable proportions of IFNγ, IL-4, IL-17 or foxp3 expressing cells (Figure 4-2A), the viability of these cells was often poor at the end of the culture period. When cells were activated continuously, viability of Th1s, Th2s and Tregs deteriorated following splitting of cells on day 3 and harvesting on day 5 or 6. However this was not observed with Th17s, which maintained viability despite continual activation for the full 5 or 6 days of culture (Figure 4-2B).

IL-2 is a cytokine which promotes T cell survival and proliferation. I therefore looked at the effects of IL-2 on viability of Th1, Th2 and Treg cultures. In addition it was possible that other unknown autocrine factors that were important for survival may have been secreted by the cells into the culture media and were being removed during the process of splitting the cells. I therefore tried ‘splitting’ the cells by transferring them with their media into larger wells and adding new media on top. However, neither of these interventions improved cell viability.
The effects of the duration of activation on in vitro differentiated CD4 subsets

A   Cells were cultured for 5 days in Th1, Th2, Th17 and Treg conditions and were stimulated with antiCD3 and anti-CD28 from days 0-3 or 0-5 before restimulation and analysis by intracellular flow cytometry on day 5.  B   Tables showing percentage expression of the phenotypic marker for each subset – IFNg for Th1s, IL-4 for Th2s, IL-17 for Th17s and foxp3 for Tregs – and percentage viability at the end of 5 days culture in cells stimulated for 3 days (black symbols) or 5 days (red symbols).
Activation induced cell death occurs when T cells are exposed to repeated TCR stimulation and this appeared likely to explain the fall in viability between day 3 and 6. In contrast to the other subsets, the viability of Th17s remained high despite 5-6 days continual stimulation, which is consistent with the finding that Th17s are less susceptible to AICD than Th1s (Yu, Iclozan et al. 2009). I therefore examined viability and phenotypic expression in Th1s, Th2s, Th17s and Tregs after removing aCD3 and aCD28 stimulation from day 3 onwards compared with stimulation for the entire period of culture. I found that removing stimulation at day 3 and adding IL-2 for the remainder of culture resulted in improved viability of the Th1, Th2 and Treg subsets, which were generally maintained at over 90%, compared with cells grown with continued stimulation (Figure 4-2B).

Removing stimulation at day 3 improved viability but was associated with a slight decrease in the percentage of IFNg expressing cells in Th1 cultures and a more marked decrease in IL4 +ve cells in Th2 cultures. When stimulation was removed from Th17 cultures at day 3 the percentage of IL17 +ve cells dropped to less than 2% by day 5 or 6. Removing stimulation from Treg cultures improved both the viability and the percentage of foxp3 +ve cells (Figure 4-2B).

Therefore, to generate cells which were both differentiated into the appropriate phenotype and sufficiently viable for adoptive transfer into mice it was clear that activation needed to be removed from Th1, Th2 and Treg cultures at day 3. For Th1 and Th2 subsets the improvement in viability came at the expense of reduced expression of IFNg and IL-4 respectively. For Th17 cells continued activation was necessary to maintain the IL-17+ve phenotype and did not worsen viability. For Tregs
both viability and phenotype were improved by withdrawal of activation at day 3.

Final culture conditions are summarised in Table 4-1.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Activation aCD3, aCD28</th>
<th>Polarising cytokines</th>
<th>IL-2 / IL-23</th>
<th>Neutralising antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>Day 0-3</td>
<td>IL-12 (5ng/ml)</td>
<td>IL-2 day 3-5</td>
<td>anti IL4</td>
</tr>
<tr>
<td>Th2</td>
<td>Day 0-3</td>
<td>IL-4 (10ng/ml)</td>
<td>IL-2 day 3-5</td>
<td>anti IFNg</td>
</tr>
<tr>
<td>Th17</td>
<td>Day 0-5</td>
<td>TGFb (2.5ng/ml)</td>
<td>IL-23 day 0-5</td>
<td>anti IL-4 anti IFNg</td>
</tr>
<tr>
<td>Treg</td>
<td>Day 0-3</td>
<td>TGFb (10ng/ml)</td>
<td>IL-2 day 3-5</td>
<td>anti IFNg</td>
</tr>
<tr>
<td>Th0</td>
<td>Day 0-3</td>
<td>Nil</td>
<td>IL-2 day 3-5</td>
<td>anti IFNg anti IL4</td>
</tr>
</tbody>
</table>

Table 4-1 Summary of final culture conditions

4.2.4 CD4 subsets generated from HNT CD4 T cells maintain ability to recognise HA antigen after in vitro differentiation

Having established conditions for optimal differentiation of CD4 subsets I applied these protocols to naive CD4+ T cells harvested from HNT mice. CD4 T cells from HNT mice express a TCR which recognises the class II restricted HNT epitope in haemagglutinin (HA). It was important that these in vitro differentiated CD4 cells retained the ability to respond to HNT peptide after 5 days of culture. I therefore compared IFNg, IL-4 and
IL-17 expression on CD4 T cell subsets after restimulation with PMA / ionomycin or peptide pulsed splenocytes (Figure 4-3). This showed that expression of IFNg and IL-4 was similar with HNT peptide to PMA/ionomycin. IL-17 expression was slightly reduced in the peptide stimulated cells. Together this demonstrates that in vitro differentiated HNT CD4+ subsets retain the ability to recognise and respond to HNT peptide.
Figure 4-3  In vitro differentiated HNT CD4 subsets retain the ability to respond to HNT peptide

CD4 subsets were generated from thy1.1+HNT CD4 cells. At the end of 5 day in vitro culture cells were restimulated with PMA/ionomycin or HNT peptide loaded splenocytes from a thy1.2+ balb/c mouse, in the presence of brefeldin A. A shows gating of Thy1.1+ve cells in mixed thy1.1/thy1.2 cultures. B shows cytokine expression profiles on restimulated Thy1.1+ cells. Representative of 3 independent experiments.
4.2.5  *In vitro differentiated Th1s and Th2s are more stable than Th17s and T regs in vitro*

Although the above conditions generated a population of cells which appeared to have differentiated into Th1s, Th2s, Th17s and Tregs, it was important to know how stable these phenotypes were once cells were removed from their differentiation conditions. I generated Th1, Th2, Th17 and Tregs and activated each subtype for 2 further days in each of the alternative differentiation conditions (Th1, Th2, Th17, Treg, Th0) as well as in media only (Figure 4-4).

This showed that Th1 cells were stable in all conditions except Th2, where IFNg expression was substantially reduced.

Th2 cells were stable in all conditions except in media only. Interestingly, despite negligible expression at day 5, increased IL-4 expression was seen after 2 further days culture in all conditions containing CD3 and CD28 stimulation, irrespective of the cytokines added.

Th17s and Tregs were less stable. A proportion of cells became IFNg and IL-4 secreting in Th1 and Th2 conditions with loss of IL-17 and foxp3 expression. Th17s required TGFb (Treg and Th17 conditions) to maintain substantial levels of IL-17 expression, whereas high levels of foxp3 expression amongst Tregs was maintained in Treg conditions only.
In vitro differentiated Th1s and Th2s are more stable than Th17s and Tregs

Th1s, Th2s, Th17s and Tregs were generated in vitro over 5 days and then cultured for a further two days in the presence of anti-CD3 and anti-CD28 in each of the alternative polarising conditions described in Table 4-1 (nil=media only). On day 7 cells were harvested and restimulated with PMA and ionomycin and analysed by FACS. Representative of two independent experiments.
4.2.6 In vivo behaviour of in vitro differentiated CD4 subsets following adoptive transfer

In order to confirm that in vitro differentiated CD4 cells survive in vivo following adoptive transfer, 5x10^5 in vitro polarised HNT Thy1.1+ CD4 cells were injected iv into non-tumour bearing Thy1.2+ mice and spleens and lymph nodes harvested 2, 7 and 21 days later (Figure 4-5A). Adoptively transferred thy1.1+CD4 cells were identified by flow cytometry (Figure 4-5B).

Survival and homing of cells to lymphoid compartments was found to vary between subsets (Figure 4-5C). Initial homing of cells to lymph nodes was highest in Th1 and Treg subsets, however by day 7 Th2s were found in lymph nodes in equivalent proportions. Th1s, Th2s and Tregs were found in equivalent quantities in the spleen at day 2, however by day 7 Tregs had started to disappear in both the spleen and lymph node. Th17s were found in a very low frequency in the spleen only at day 2 and had almost completely disappeared in all compartments thereafter. By day 21, the numbers of Th1s had declined with only Th2s surviving long term, at levels unchanged from that seen at day 2.

In summary persistence of adoptively transferred cells in the spleen and lymph node varied between different subsets with Th17s essentially disappearing post transfer and Th2s displaying the most longevity.
Figure 4-5  Recovery of in vitro differentiated CD4 subsets following adoptive transfer into naïve mice

Thy1.1+ Th1s, Th2s, Th17s and Tregs were differentiated in vitro and 5x10⁵ cells were injected iv into recipient thy1.2+ balb/c mice. Harvests were performed 2, 7 and 21 days later as in schedule in A. Gating on CD4+Thy1.1+ve cells is shown in B. Recovery of CD4+Thy1.1+ve cells from lymph nodes and spleen is displayed in C. Experiment performed once.
CD62L is a cell surface marker expressed by naive T cells and central memory subsets which facilitates entry of circulating T cells into lymph nodes. I therefore analysed expression of CD62L on the differentiated CD4 subsets at the end of the in vitro differentiation period (Figure 4-6). This showed that the level of CD62L expression was consistently highest on Th1s and Tregs, the two subsets found in the greatest frequency in lymph nodes at day 2, suggesting that differences in baseline CD62L expression may explain the variable initial lymph node trafficking seen between subsets.
Figure 4-6  Initial homing of CD4 subsets to lymph nodes correlates with expression of CD62L

A CD62L expression was analysed on in vitro differentiated CD4 subsets at the end of 5 days culture. Results from 3 independent cultures are shown in B. The recovery of thy1.1+CD4 cells from the experiment described in Figure 4-5 is shown in C for comparison.
4.2.7 Survival and behaviour of CD4 subsets following adoptive transfer into tumour bearing mice

It was important to determine how the presence of tumour affected the behaviour of adoptively transferred CD4 subsets. The presence of AB1-HA tumour could potentially provide HA antigen and therefore drive proliferation of HNT CD4 cells but malignancy can also invoke a number of immunosuppressive mechanisms which might impair survival or proliferation of the adoptively transferred cells.

CD4 subsets were generated from thy1.1+ HNT cells and adoptively transferred into Thy1.2 mice bearing 8 day old AB1HA tumours. Organs were harvested 2 and 7 days after transfer. It was not possible to examine later time points due to outgrowth of tumours. Similar to that seen in non-tumour bearing mice, Tregs and Th1s were found in the greatest frequency in lymph nodes at day 2 (Figure 4-7A). In addition, the presence of tumour favoured accumulation of Th1 and Treg subsets in the draining node compared to the non draining node, whereas no difference was seen for Th2s and Th17s. The frequency of Tregs in the DLN two days after adoptive transfer was now four-fold higher than that seen for Th1s. This suggests that the presence of AB1HA tumour provides a favourable environment for initial accumulation or proliferation of HA specific Tregs in the DLN.

At day 2 Tregs were seen at the greatest frequency in all compartments, however consistent with results in naive mice, they had disappeared by day 7 (Figure 4-7B). In contrast Th1s and Th2s expanded in the DLN or spleen between day 2 and day 7. Since expansion of these subsets had not been observed in non tumour bearing mice during
this period, proliferation could be attributed to the presence of the HA expressing
tumour.

Again the quantity of Th17s that could be found at any time point was around 10 fold
lower than that seen in other subsets indicating that the presence of cognate antigen
did not improve the survival of these cells. It was possible that Th17s may be homing
to peripheral tissues rather than lymphoid compartments. I therefore harvested lungs
from mice alongside other organs and analysed recovered cells by flow cytometry
(Figure 4-7C). The frequency of each subset in the lung was similar to that seen in the
spleen, indicating that there was no preferential accumulation of Th17s in the lung.
Since Th17s were activated for 5 rather than 3 days, I also tried removing activation
from Th17s after 3 days, in case the Th17s were undergoing activation induced cell
death after adoptive transfer. However this did not improve recovery of Th17 cells 2
days after adoptive transfer (data not shown).

These results indicate that the presence of AB1HA tumour favours early accumulation
of Tregs in the DLN and spleen (but does not prevent their disappearance by day 7),
promotes expansion of adoptively transferred Th1 and Th2s between days 2 and 7, but
does not improve survival of Th17s.
Figure 4-7  Recovery of in vitro differentiated CD4 subsets following adoptive transfer into tumour bearing mice.
Thy1.2+ balb/c mice bearing 8 day old AB1-HA tumours received adoptive transfer of 5x10^5 in vitro differentiated Thy1.1+ Th1s, Th2s, Th17s or Tregs. Harvests were performed 2 and 7 days later and recovered thy1.1+CD4 cells were identified by FACS, as shown in Figure 4-5B. A Initial accumulation of thy1.1+CD4 cells in DLN and NDLN 2 days after adoptive transfer, statistical comparisons performed using paired T test. B Recovery of thy1.1+ CD4 cells from DLN, NDLN and spleen at 2 and 7 days after transfer. Statistical comparisons performed using unpaired t test with significant differences between values obtained on day 2 and day 7 for each organ shown. A and B show results from two independent experiments (total 6 mice per group). C Recovery of thy1.1+ CD4s from lung tissue 2 days after adoptive transfer.

4.2.8 Th1s and Th2s are able to infiltrate tumours

Although Th1 and Th2 cells were found to proliferate in lymphoid compartments in the presence of AB1HA tumour, this did not necessarily mean that these cells were able to infiltrate tumours and effect anti-tumour activity. I therefore looked for the presence of adoptively transferred thy1.1 CD4 cells in tumours harvested 7 days after adoptive transfer. Single cells suspensions were prepared and cells analysed by flow cytometry (Figure 4-8A). The proportion of tumour infiltrating CD4 cells which consisted of adoptively transferred Thy1.1 HNT cells was determined. In addition, in order to account for any effects the HA specific CD4 cells may have had on the total infiltrating CD4 population, the proportion of infiltrating thy1.1+ve cells was also expressed as a percentage of total tumour cells, as gated from forward and side scatter plots (
This showed that adoptively transferred Thy1.1+ve Th1s infiltrated the tumour in the greatest abundance, composing between 3-20% of all tumour infiltrating CD4s. Thy1.1+ve Th2s were also found in the tumour at a slightly lower proportion but Thy1.1+ve Tregs and Thy1.1+ve Th17s were not detected.

**Figure 4-8 A)**

Tumour Thy1.1+ve CD4s

<table>
<thead>
<tr>
<th>Th1</th>
<th>Th2</th>
<th>Th17</th>
<th>Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

**Figure 4-8 B)**

% Total CD4s

% Total cells

**Figure 4-8 Ability of CD4 subsets to infiltrate tumours**

Tumours from mice treated as described in Figure 4-7 were harvested 7 days after adoptive transfer and recovered cells analysed by flow cytometry. A shows example of gating – total cells were determined from forward and side scatter plots such that all lymphocytes were included but events lying on the axis were excluded, and thy1.1+ CD4 cells were identified as shown. B Recovery of thy1.1+ CD4 cells from tumours, expressed both as a percentage of total tumour infiltrating CD4 cells and as a percentage of total cells.
4.2.9 Stability of polarised phenotype in vivo

The phenotypic expression of Thy1.1+ve HNT CD4 subsets after adoptive transfer into tumour bearing mice was determined by harvesting organs and restimulating cells with PMA and ionomycin ex vivo. Expression of IFNg, IL-4, IL-17 and foxp3 on recovered cells was analysed by intracellular staining and flow cytometry.

Expression of IFNg, IL-4, IL-17 and foxp3 on recovered Thy1.1+ Th1s, Th2s, Th17s and Tregs is shown in Figure 4-9A. Compared to levels of IFNg expression on the cells prior to adoptive transfer, Th1s showed a small decrease in the proportion of IFNg-expressing cells by day 2 suggesting either early death of some overstimulated cells through activation induced cell death or loss of IFNg expression by some cells (Figure 4-9B). However between day 2 and day 7 the proportion of IFNg secreting cells remained stable, indicating that in vitro differentiated Th1s maintain their phenotype in vivo.

Interestingly, when Thy1.1+ve Th2s were recovered there was a marked increase in the proportion of cells which expressed IL-4 compared to that seen at the end of their 5 day in vitro culture before transfer. This mirrors the in vitro data described in section 4.2.5, where restimulation of cells for a further two days increased the percentage of IL-4+ve cells. Unlike Th1s, IL-4 expression was much higher in the spleen than the DLN at day 2, suggesting either that IL-4 negative cells preferentially track to lymph nodes or that IL-4 expression was being suppressed in the DLN. Between days 2 and 7 levels of IL-4 expression declined in the spleen but were maintained in the DLN.
Although few Thy1.1+ve Th17s were found at any time point, those that were detectable did contain a proportion of IL-17 secreting cells, and this proportion was higher in the spleen than the DLN.

Recovered Thy1.1+ve Tregs showed an initial loss of foxp3 positive cells. Levels of foxp3 expression were stable thereafter.

Overall these results indicate that for all subsets, the proportion of recovered cells expressing the phenotype imposed in vitro was generally comparable, or increased in the case of Th2s, to that seen pre-transfer. This suggests that the phenotype imposed during in vitro culture is maintained by a similar proportion of cells in vivo.
Figure 4-9  Ex vivo phenotypic expression on CD4 subsets recovered 2 and 7 days after adoptive transfer

Thy1.1+ CD4 cells were recovered 2 and 7 days after adoptive transfer into tumour bearing mice, as described in Figure 4-7, and restimulated ex vivo with PMA and ionomycin. Thy1.1+CD4+ cells were identified by flow cytometry as shown in Figure 4-5B.  A  Expression of IFNg, IL-4, IL-17 and foxp3 on Thy1.1+CD4+ cells.  B  Percentage of recovered Th1s, Th2s, Th17s and Tregs expressing IFNg, IL-4, IL-17 and foxp3 respectively, compared to baseline (pretransfer) levels.  Shows results from 2 independent experiments (n=6 at each timepoint) (3 data points missing from Th1, Th2 and Th17 day 2 graphs due to failed restimulation).
4.2.10 Some adoptively transferred Th2s and Th17s become ‘Th1s’ after 7 days in vivo in tumour bearing mice

Although a proportion of in vitro differentiated CD4 cells maintained expression of their phenotypic markers in vivo, it was clear from this experiment that 7 days after adoptive transfer, amongst recovered thy1.1+ve Th2, Th17 and Treg populations there was an additional population of IL-4, IL-17 and foxp3 negative cells which had become IFNγ secreting (Figure 4-10A). In contrast there was no detectable IL-4, IL-17 or foxp3 expression on recovered thy1.1+ve Th1s (data not shown).

Prior to adoptive transfer the proportion of IFNγ secreting cells in Th2, Th17 and Tregs was low and typically between 0-5%. At day 2 levels of IFNγ were still low in the DLN and the spleen, however by day 7 around 12% of recovered thy1.1+ve ‘Th2s’ and 25% of recovered thy1.1+ve ‘Th17s’ were IFNγ positive in the spleen. When tumour infiltrating thy1.1+ve ‘Th2s’ were examined the proportion of IFNγ secreting cells rose further to almost 40% (Figure 4-10B).

These results indicated the tumour environment in this model favours the development of an IFNγ+ ‘Th1’ phenotype amongst a proportion of the adoptively transferred CD4 cells.
Cells were recovered from DLN, spleen and tumour 2 and 7 days after adoptive transfer of 5x10^5 in vitro differentiated Thy1.1+ CD4 subsets and restimulated ex vivo with PMA and ionomycin, as described in Figure 4-7. Thy1.1+CD4+ cells were identified by flow cytometry as shown in Figure 4-5B. A Expression of IFNγ on thy1.1+ Th2, Th17 and Treg subsets recovered from DLN and spleen was determined by flow cytometry. B Expression of IFNγ on tumour infiltrating thy1.1+ Th1s and Th2s 7 days after adoptive transfer, shows analysis of pooled tumours from 3 mice.
4.3 Discussion

The first part of this chapter describes the optimisation of protocols for differentiating naive CD4 T cells into Th1, Th2, Th17 and Treg subsets. Initially I was mainly concerned with maximising the proportion of cells which expressed the appropriate phenotype. Based on reports in the literature I found that IL-12 induced Th1 differentiation, IL-4 induced Th2 differentiation, IL-6 and low dose TGFb induced Th17 differentiation and high dose TGFb induced Treg differentiation. In addition I found that adding neutralising antibodies to IFNg and IL-4, where appropriate, further increased the proportion of phenotype positive cells.

To activate cells I used anti-CD3 and anti CD28 antibodies. This method is well described (Zhang, Zhang et al. 2003; Harrington, Hatton et al. 2005; Nurieva, Yang et al. 2009), although other methods of activating cells with peptide loaded antigen presenting cells have also been commonly reported (Nishimura, Iwakabe et al. 1999; Park, Li et al. 2005; Lexberg, Taubner et al. 2008; Martin-Orozco, Muranski et al. 2009). I found that the duration of the activating stimulus had a profound effect on the phenotypic expression and viability of in vitro differentiated cells, consistent with cells undergoing activation induced cell death with prolonged activation. For Th1s and Th2s, continuous activation for 5 days produced high frequencies of IFNg and IL-4 positive cells respectively but at the expense of cell viability. To preserve viability I had to settle for a shorter activation period with a reduced frequency of phenotype positive cells at the end of the culture period. Th17s however appeared more resistant to activation induced cell death and indeed required continual activation to maintain their phenotype.
These results suggest that the culture conditions can have a marked effect on the quality of the in vitro differentiated CD4 cells which are produced. As a consequence comparisons between studies which use markedly different protocols for in vitro activation and differentiation of CD4 cells need to be made cautiously. Other investigators have also reported removing activation from aCD3, aCD28 activated Th1 and Th2 cultures after 3 days (Zhang, Zhang et al. 2003) whereas this was not reported for cells activated by peptide loaded irradiated APCs (Park, Li et al. 2005; Lexberg, Taubner et al. 2008; Martin-Orozco, Muranski et al. 2009). It could be that the strength of the TCR stimulus from peptide loaded APCs is weaker or declines naturally during culture such that removal of the activating stimulus is not necessary.

To investigate the degree of plasticity of in vitro differentiated CD4 cells I tried to repolarise them into each of the other subsets. This showed that Th1s and Th2s were more stable than Th17s and Tregs. This is consistent with previous studies and is consistent with the theory that Th1s and Th2s are a more terminally differentiated CD4 cell than Th17s or inducible Tregs (Murphy and Stockinger 2010). Interestingly however when I examined the stability of the CD4 cells in vivo in tumour bearing mice, I found that Th1s were highly stable but that after 7 days a proportion of recovered thy1.1 cells from Th2, Th17 and Treg cultures had become IFNg secreting. This was most pronounced amongst tumour infiltrating cells. This suggests that the microenvironment in AB1-HA tumour bearing mice favours Th1 polarisation. Since the transferred population contained both phenotype positive and negative cells, it is unclear whether the Th2, Th17 or Treg cells which had become IFNg +ve had arisen from the original positive or negative population.
Although the populations of each CD4 subset at the end of the culture period contained both phenotype positive and negative cells, it is interesting to note that the negative population still display some features of fully differentiated cells. In the Th2 cultures, despite >99% of cells being IL-4 negative at the end of 5 days in vitro differentiation, these cells were still highly resistant to repolarisation into other subtypes in vitro. Furthermore, higher frequencies of IL-4+ cells were detected after a further 48 hours exposure to TCR stimulation, which was observed following two days in vitro culture and after recovery ex vivo. These findings suggest that despite the lack of IL-4 expression initially, these cells still display some degree of Th2 differentiation.

In addition, the Th17 cultures contained around 60-70% IL-17 negative cells yet recovery of Th17s following adoptive transfer was over ten-fold lower than other subtypes, suggesting that the survival and/or homing of the IL-17 negative population mirrors that of the IL-17 positive cells rather than the negative population from other subtypes.

These findings suggest that the population of negative cells in CD4 subset cultures are not simply undifferentiated CD4 cells but in a number of respects behave similarly to their phenotype positive counterparts, despite the lack of expression of the signature cytokine. It is possible, therefore, that intracellular cytokine detection by flow cytometry may be an insensitive method of detecting polarisation of some CD4 subsets.

Following adoptive transfer into mice, trafficking and survival of in vitro differentiated CD4 cells differed between subsets. Initial homing of cells to lymph nodes was highest in Th1s and Tregs. This can be explained by their higher expression of CD62L, a cell surface marker expressed by naive and central memory cells that facilitates entry into...
lymph nodes. In contrast CD62L expression on Th17s was consistently low and was variable on Th2s. The presence of AB1HA tumour favoured the early accumulation/expansion of Tregs in the DLN and spleen over the other subsets, suggesting that the tumour environment may promote initial Treg homing, survival and/or proliferation over effector subsets.

In naive mice Th2s displayed the greatest longevity but other subsets had started to decline in frequency by day 7. However, the presence of an HA expressing tumour led to the expansion of antigen specific Th1s and Th2s in vivo, with substantial tumour infiltration by the Th1 subset. This confirms that the observed ability of HA specific CD4 subsets to respond to HA peptide in vitro is mirrored in vivo by expansion of cells in the presence of HA antigen. In contrast Tregs had disappeared 7 days after adoptive transfer in both naive and tumour bearing mice. Surprisingly I could recover very few Th17s from lymph nodes and spleens at any timepoint following adoptive transfer. This was despite these cells being over 90% viable prior to transfer. Furthermore removing the activating stimuli from Th17 culture at day 3 did not improve recovery of Th17s, suggesting activation induced cell death was not the cause of the lower recovery compared to other subsets. In addition I looked for Th17s in peripheral tissues and could not find evidence of preferential homing of Th17s to other, non-lymphoid sites.

These results demonstrate that the behaviour of in vitro differentiated CD4s following adoptive transfer differs markedly between subsets. As such, differential in vivo activity of in vitro differentiated CD4s may not only result from the specific set of cytokines produced by each subset but may also be due to differential homing and survival. The failure to find Th17s after adoptive transfer is striking and contrasts to
other studies where Th17s were found to have a survival advantage over Th1s in lymphopenic hosts (Muranski, Boni et al. 2008) and a similar survival capacity to Th1s in non-lymphopenic mice (Martin-Orozco, Muranski et al. 2009). It is unclear why Th17s did not survive in this model, however these studies used peptide loaded APCs to stimulate cells during in vitro culture as well as different mouse strains. Balb/c mice are reported to favour Th2 differentiation when compared to C57BL/6 mice (Arimura, Kato et al. 2002), so it is possible that T cells from these strains may respond differently to in vitro polarisation protocols.

In summary, this chapter demonstrates that naive HA specific CD4 cells can be polarised in vitro into Th1s, Th2s, Th17s and Tregs. These subsets demonstrate variable homing, survival and plasticity in vivo but Th1s and Th2s are able to expand in vivo and infiltrate AB1HA tumours. Based on these results I went on to examine the anti-tumour activity of these cells against AB1-HA tumours.
Using in vitro differentiated CD4 cells to treat tumours
5.1 Introduction

Having established protocols for in vitro differentiation of HA specific CD4 subsets, I next examined whether these cells had anti-tumour activity in vivo against AB1-HA tumours. CD4 cells can mediate anti-tumour effects through a number of mechanisms, but it is the provision of help to tumour specific CD8 T cells which is widely regarded as their primary mode of action (Lake and Robinson 2005; Kennedy and Celis 2008). Therefore it was important to ensure that there was an adequate precursor frequency of HA specific CD8s available so that the help provided by HA specific CD4s would be apparent, and also so that any differences in outcome between mice receiving different CD4 subsets could be attributed to the CD4 cells themselves and not due to differences in the availability of CD8 effectors.

CL4 mice express a T cell receptor which recognises a class I restricted HA epitope. I therefore opted to co-transfer HA specific CD8 T cells harvested from a naive thy1.1+ CL4 mouse alongside the HNT CD4 cells. In early experiments I transferred unsorted CL4 lymphocytes, however in later experiments, due to a limited supply of CL4 mice, I sorted CD8 T cells from CL4 lymph nodes and spleens through magnetic column separation and transferred an equivalent number of CD8 T cells. Since thy1.1+ CL4 CD8s could be identified ex vivo with flow cytometry, I was able to track the activity of the HA specific CD8 cells and hence determine the effects of cognate help provided by each HA specific CD4 subset to HA specific CD8s.

I first examined the anti-tumour activity of in vitro differentiated CD4 cells in a prophylactic treatment setting, where HA specific T cells were injected prior to inoculation with AB1-HA. Due to the absence of established tumour-induced
immunosuppression and the lowest tumour load, this could be considered a ‘best case scenario’, where anti-tumour activity of adoptively transferred T cells is likely to be maximal.

I then looked at the effects of adoptive transfer of CD4 subsets against established tumours. In chapter 3, I demonstrated that gemcitabine causes lymphodepletion followed by homeostatic proliferation and leads to a reduction in tumour infiltrating Tregs 7 days after treatment. I hypothesised that treatment with gemcitabine would provide a favourable window for adoptive transfer. The plasma half life of gemcitabine in mice is around 30 minutes (Shipley, Brown et al. 1992), therefore I opted to transfer cells 24 hours after gemcitabine administration, to ensure the transferred cells were not affected by the drug.
5.2 Results

5.2.1 Titrating the number of HA specific CD8 T cells for co-transfer with in vitro differentiated CD4 subsets

In order to ensure that there were an adequate number of HA specific CD8 T cells in our tumour model, such that the availability of tumour specific CD8 T cells was not going to limit the effects of the adoptively transferred CD4 subsets, HA specific CD8 T cells from a naive CL4 mouse were co-transferred alongside the HNT CD4 subsets.

Transfer of large numbers ($10^7$) of naive CL4 lymphocytes has been shown to mediate tumour rejection in a high proportion of mice in the AB1-HA model (Marzo, Lake et al. 1999). We therefore titrated the number of transferred CL4 cells to try to find the tipping point at which there were likely to be enough HA specific CD8 cells available to mediate the effects of the adoptive transferred CD4 cells, but not so many that they would themselves substantially delay tumour growth. CL4 lymphocytes were injected intravenously alongside naive HNT lymphocytes and 1 day later mice were challenged with AB1-HA. There were no cures with these treatments, but in mice that received both lower ($5 \times 10^4$) and higher ($5 \times 10^5$) numbers of HNT cells, tumour growth was delayed when more than $1 \times 10^6$ CL4s were used (Figure 5-1). With $1 \times 10^6$ CL4s a small but significant prolongation of survival was seen. Therefore in future treatment experiments we elected to co-transfer $1 \times 10^6$ CL4 lymphocytes alongside the in vitro differentiated CD4 subsets.
Figure 5-1  Titrating numbers of HA specific CD8s for co-transfer with HA specific CD4s

Mice were injected i.v. with naive HNT CD4 cells in combination with CL4 lymphocytes on day -1 and challenged with 5x10^5 AB1-HA cells at day 0. Tumour growth and survival curves are shown. Kaplan Meier plots were analysed by log rank test and significance is shown in comparison to mice receiving HNT cells only.
5.2.2 Th1s mediate tumour rejection in a prophylactic treatment model

The anti-tumour activity of in vitro differentiated CD4 subsets was first examined in a prophylactic treatment setting, as this could be considered the most optimal situation to see anti-tumour activity. Th1, Th2, Th17, Treg and Th0 subsets were generated in vitro (Figure 5-2A) and were injected intravenously with CL4 lymphocytes. 1 day later mice were challenged with AB1HA. Survival and tumour growth was compared to control mice that received CL4 cells alone (Figure 5-2B). This showed that only Th1 cells provided a significant survival advantage, with 2 of 5 mice rejecting tumours and surviving long term. In addition, in the mice which did not survive, only Th1s led to a significant delay in rate of tumour growth (Figure 5-2B). Although no significant differences in growth rates were observed with any of the other CD4 subsets, tumours were noted to grow most quickly in mice that received adoptive transfer of Tregs.
Figure 5-2  Prophylactic treatment with HA specific CD4 subsets

A  Th1, Th2, Th17, Treg and Th0 HNT CD4 subsets were differentiated in vitro.  B  Mice received intravenous adoptive transfer of 5x10^7 HNT cells and 1x10^6 CL4 lymphocytes, 1x10^6 CL4 lymphocytes alone or no adoptive transfer on day -1 and inoculation of 5x10^5 AB1-HA subcutaneously on day 0.  Survival curves and tumour growth curves in non surviving mice are shown.  Significance compared to mice receiving CL4 cells alone.  5 mice per group.
5.2.3 Adoptive transfer of in vitro differentiated CD4 subsets does not improve outcomes following treatment with gemcitabine

Experiments described in chapter 3 demonstrated that a single dose of gemcitabine caused transient lymphodepletion, reduced the frequency of regulatory CD4 T cells within the tumour and substantially increased CD8 tumour infiltration. However this was not sufficient to cure tumours in the majority of cases. Therefore examined whether adoptive transfer of in vitro differentiated CD4 subsets, in conjunction with tumour specific CD8 T cells, in the immediate post chemotherapy setting could enhance the anti-tumour immune response, and whether this would be sufficient to cure mice with established tumours.

Mice bearing 9 day old AB1HA tumours were treated with a single dose of gemcitabine. 24 hours later mice received an intravenous injection of in vitro differentiated Th1, Th2, Th17, Treg or Th0 cells, plus CL4 cells (Figure 5-3A). Control groups received CL4 cells alone or no adoptive transfer. In contrast to the prophylactic setting, adoptive transfer of tumour specific CD4 subsets did not improve survival (Figure 5-3B) or delay tumour growth (data not shown) compared to mice that received transfer of CL4 cells alone. In all groups a small percentage of mice were cured of their tumour, however this appeared to be simply an effect of gemcitabine treatment.

Since gemcitabine on its own was capable of eradicating tumours, I repeated this experiment with a reduced the dose of gemcitabine. However, transfer of CD4 subsets on day 12 following a lower dose of gemcitabine on day 9 still had no effect on survival or tumour growth (Figure 5-3C).
A Th1, Th2, Th17, Treg and Th0 HNT CD4 subsets were differentiated in vitro. 

B + C Mice bearing 9 day old AB1-HA tumours were treated with 240ug/g gemcitabine (B) or 120ug/g gemcitabine (C) followed by i.v. adoptive transfer on day 10 (B) or day 12 (C) of 5x10⁵ HNT cells and 1x10⁶ CL4 lymphocytes, 1x10⁶ CL4 lymphocytes alone or no adoptive transfer. Between 5-7 mice per group.

Figure 5-3 Treatment of established tumours with gemcitabine followed by adoptive transfer of CD4 subsets
5.2.4 Gemcitabine does not deplete cells transferred 24 hours later

Although the plasma half life of gemcitabine is around 30 minutes, the half life in tissues can be longer (Shipley, Brown et al. 1992). Thus it was important to ensure that the adoptively transferred cells were not being killed by residual gemcitabine not cleared from tissues at the time of transfer. I therefore injected non-tumour bearing mice with a single dose of 240 ug/g of gemcitabine 1 day, 2 days and 3 days prior to adoptive transfer of in vitro differentiated Th1s and counted thy1.1+CD4 cells recovered from spleens 48 hours later (Figure 5-4). This showed that cells transferred 24 hours after gemcitabine were not being depleted compared to control mice.

![Figure 5-4](image)

**Figure 5-4** Effects of timing of gemcitabine on recovery of adoptively transferred cells

Mice were treated with 240ug/g gemcitabine 1, 2 or 3 days before receiving adoptive transfer of $1 \times 10^6$ in vitro differentiated thy1.1+ HNT Th1s. 2 days later spleens were harvested and thy1.1+ CD4 cells identified by flow cytometry.
5.2.5 Increasing the number of adoptively transferred CD4 subsets or tumour specific CD8 T cells does not improve outcomes

In the previous experiments, because of difficulties generating large numbers of polarised CD4 cells in vitro, mice were treated with 5x10^5 HNT CD4 cells. This is fewer than many other investigators have used in similar experiments (Nishimura, Iwakabe et al. 1999; Muranski, Boni et al. 2008), where up to 2x10^7 cells were transferred. Since the effects of adoptive transfer in treatment experiments had been minimal, these experiments were repeated using an increased number of CD4 cells (Figure 5-5). Mice were treated in both the prophylactic and the post-gemcitabine situation with 2.5x10^6 in vitro differentiated HNT CD4 cells and 1x10^6 CL4 cells. However, even though cell number was increased five-fold, no improvement in numbers of surviving mice was observed. Surprisingly, we found that the number of survivors from the Th1 treated group in the prophylactic experiment was less than that seen in the original experiment (Figure 5-5B). Despite this, the same trend was observed – that only Th1s were able to significantly improve survival in the prophylactic setting compared to mice who received CL4 cells alone.

In the post-gemcitabine setting, adoptive transfer of five-fold more CD4 cells did not generate any long term survivors, but there was a small delay in the rate of tumour growth with Th1 and Th2 cells (p<0.05), suggesting some limited anti-tumour activity (Figure 5-5C).
A) Th1, Th2, Th17, Treg and Th0 HNT CD4 subsets were differentiated in vitro.  
B+C) Mice received adoptive transfer of 2.5x10^6 HNT CD4 cells with 1x10^6 CL4 lymphocytes 1 day before tumour inoculation (B), or 1 day after treatment with 240ug/g gemcitabine (C). 6 mice per group.  Graphs show survival curves and tumour growth in non-surviving mice.  Significance compared to mice receiving CL4 cells alone.
Since increasing the number of tumour specific CD4 cells did not improve overall survival, it was important to ensure that there were no other factors which could have been limiting the activity of the adoptively transferred CD4 cells. Although I had previously attempted to titrate the number of CL4 lymphocytes transferred in conjunction with naive HNTs, it was still possible that inadequate numbers of tumour specific CD8 T cells could explain the lack of effectiveness of the CD4 cells. Whether this might be the case was examined by repeating the prophylactic treatment experiment using only the Th1 subset. Increasing numbers of CL4 lymphocytes were co-transferred in order to ascertain if there was a CD8 dose dependant effect (Figure 5-6). With low (5x10^5) and high (1x10^6) numbers of Th1s transferred, increasing the number of CL4 cells by a factor of 10 had no significant effect on survival. In all groups we found that, similar to previous experiments, approximately 40% of mice rejected tumour. This suggests that the availability of HA specific CD8 T cells was not a limiting factor in the model.
Figure 5-6  Treatment experiments using increased numbers of HA specific CD8s

Mice received adoptive transfer of HNT Th1s and CL4 lymphocytes in the doses indicated at day -1 and were challenged with $5 \times 10^5$ AB1-HA on day 0.
5.2.6 In vitro differentiated HA specific CD4 subsets fail to provide help to HA specific CD8s in mice with established tumours

The above experiments suggested that the anti-tumour activity of HA specific CD4 and CD8 T cells was not limited by the absolute number of cells transferred. I next investigated whether there could be a functional problem with the cognate help provided in vivo by the HA specific CD4 cells to HA specific CD8 T cells. Thy1.1+ HNT in vitro differentiated CD4 subsets were transferred along with Thy 1.1+ CL4 lymphocytes into mice bearing established 9 day tumours. Organs were harvested and recovered cells were analysed by flow cytometry 7 days after adoptive transfer (Figure 5-7A). The effects of each HA specific CD4 subset on recovery of HA specific Thy1.1+CD8+ T cells could thus be determined and compared to mice that received CL4 cells without HNT cells (Figure 5-7B).

Similar to the experiments described in chapter 4, at 7 days after adoptive transfer Th1s, Th2s and Th0s could be recovered from DLN, spleen and tumour whereas Th17s and Tregs were not found at this timepoint. It was also clear that compared to the group that received no CD4 cells (Nil), adoptive transfer of Th1s, Th2s, Th17s or Th0s did not have any effect on the numbers of HA specific CD8 T cells recovered from the draining lymph node, spleen or tumour. This suggests that these HA specific CD4 cells did not help cognate CD8 T cells to expand within lymphoid organs or to infiltrate tumours.

In contrast, transfer of Tregs alongside CL4s led to a significant reduction in CL4 frequency in the spleen and tumour, but not the DLN, 7 days after transfer. This was despite the fact that the Tregs themselves had disappeared by this timepoint.
Figure 5-7  The effects of HA specific CD4 help on recovery ex vivo of HA specific CD8s
Mice bearing 9 day old AB1-HA tumours received adoptive transfer of $1 \times 10^6$ CL4 lymphocytes with $5 \times 10^5$ in vitro differentiated HNT Th1s, Th2s, Th17s, Tregs, Th0s or no CD4s. 7 days later DLN, spleen and tumour were harvested and thy1.1+ CD4 and CD8 cells identified by flow cytometry. A gating on thy1.1+CD4 and thy1.1+CD8 cells in DLN and tumour. B Recovery of adoptively transferred thy1.1+CD4 cells and thy1.1+CD8 cells, expressed as a percentage of total CD4s/CD8s and additionally for tumour samples, as a percentage of total cells. * denotes significance at $p<0.05$ compared to mice receiving no CD4s (Nil).

In addition to analysing the effects of CD4 subsets on cognate CD8 numbers, I also looked to see if the provision of CD4 help could be altering the function of HA specific CD8 cells. To do this I looked for expression of a positive marker of activation, ICOS, and expression of a negative regulator of function, PD-1 on recovered Thy1.1+ve CD8 cells (Figure 5-8A). The frequency of ICOS+ thy1.1+CD8 was very high in the DLN and tumour, between 80-100%, consistent with the cells being activated by the presence of HA antigen. ICOS expression was lower in the spleen. The frequency of ICOS+ thy1.1+CD8s was not affected by the presence of any of the CD4 subsets, indicating that they had not changed the activation status of HA specific CD8 cells (Figure 5-8B).

Expression of PD-1 on Thy1.1+ve CD8 cells was very high in the tumour, but was expressed at a much lower level in the DLN and there was minimal expression in the spleen (Figure 5-8B). Expression of PD-1 on HA specific (thy1.1+ve) tumour infiltrating CD8 cells was substantially higher than on the endogenous (thy1.1-) CD8 cells (Figure 5-8C). This suggests that it is the HA expression within the tumour microenvironment which promotes expression of PD-1 on HA specific CD8 cells. Again, the presence of HA specific CD4 subsets did not alter PD-1 expression on Thy1.1+ve CD8s in any compartment.
Figure 5-8  The effects of HA specific CD4 help on ICOS and PD-1 expression on HA specific CD8s
Mice bearing 9 day old AB1-HA tumours received adoptive transfer of 1x10^6 CL4 lymphocytes with 5x10^5 in vitro differentiated HNT Th1s, Th2s, Th17s, Tregs, Th0s or no CD4s (Nil). 7 days later DLN, spleen and tumour were harvested and expression of ICOS and PD-1 on thy1.1+ CD8 cells was examined by flow cytometry. Thy1.1+CD8+ cells were identified as shown in Figure 5-7A. A Gating of ICOS and PD-1 on thy1.1+ CD8 cells, on thy1.1- CD8 cells and on FMO control. B Effects of different HNT CD4 subsets on ICOS and PD-1 expression on thy1.1+ CD8 cells recovered from DLN, spleen and tumour. C Expression of ICOS and PD-1 on endogenous (thy1.1-) cells.
5.3 Discussion

The experiments described in this chapter tested the hypothesis that adoptive transfer of HA specific in vitro differentiated CD4 subsets alongside HA specific CD8 cells could enhance rejection of an HA expressing tumour. The only situation in which any of the treatment groups caused permanent rejection of tumour was with Th1s cells in the prophylactic setting. Amalgamating all experiments which used variable numbers of HA specific CD4 and CD8 cells, 15 out 43 (35%) mice treated prophylactically with Th1 cells survived long term, whereas we saw no survivors in mice who received any of the other subsets. Increasing either the quantity of transferred CD4 or CD8 cells did not increase numbers of survivors suggesting that cell number was not a limiting factor.

The finding that Th1s possess anti-tumour activity is well established. Adoptive transfer of large numbers ($10^7$) of Th1 polarised tumour specific CD4 cells caused rejection of established subcutaneous tumours in one model (Nishimura, Iwakabe et al. 1999). This study also showed that in vitro differentiated Th2 cells can have anti-tumour activity, although Th2 cells are usually considered to be a suppressive subtype in tumour immunity (De Monte, Reni et al. 2011). In contrast to my findings, in vitro differentiated Th17 cells have recently been shown to be superior at mediating tumour rejection than Th1s in a subcutaneous melanoma model (Muranski, Boni et al. 2008) and in a melanoma lung metastasis model (Martin-Orozco, Muranski et al. 2009). The lack of efficacy of the Th17s in our model is not unexpected given that they were only found in low frequencies in lymphoid and non-lymphoid organs after adoptive transfer (section 4.2.7). In addition, treatment models involving Th17 cells have used preconditioning irradiation to induce complete lymphodepletion prior to adoptive
transfer (Muranski, Boni et al. 2008). In a lymphopenic environment, Th17s have been shown to convert into an IFNγ-secreting ‘Th1-like’ cell (Nurieva, Yang et al. 2009), which is consistent with the finding that the anti-tumour activity of Th17s in lymphopenic mice was critically dependant on IFNγ and not IL-17 (Muranski, Boni et al. 2008). It is possible that Th17 cells in this model may have better survival and anti-tumour activity if complete lymphablation had been induced prior to adoptive transfer.

In the post gemcitabine setting, there was little effect from adoptive transfer of CD4 subsets. There was no improvement in numbers of mice surviving long term and even when high numbers of cells were transferred, there was only a small delay in tumour outgrowth seen with Th1 and Th2 subsets only. As such experiments described in this chapter reject the hypothesis that adoptive transfer of in vitro differentiated CD4 subsets can mediate rejection of established tumours in the post gemcitabine setting.

I therefore investigated possible reasons for the lack of efficacy of in vitro differentiated CD4 subsets against established tumours. Importantly, I excluded the possibility of any direct toxicity from residual gemcitabine on the adoptively transferred cells. In chapter four I demonstrated that in vitro differentiated Th1s and Th2s survived in vivo, expanded between days 2 and 7 post adoptive transfer, and were able to infiltrate established tumours. Additionally the experiments described in this chapter demonstrate that increasing the number of cells does not improve outcomes. It therefore seemed unlikely that an insufficient number of cells or an inability to infiltrate the tumour was limiting. As such, it was important to consider other reasons which may have explained the poor efficacy of the adoptively transferred cells against established tumours. I therefore looked to see if there was a
functional problem with the help these cells were able to provide to HA specific CD8s. This experiment showed that none of the helper CD4 subsets appeared to be able to provide help to cognate CD8s. I found no evidence of increased expansion/survival of CD8s in any compartment compared to mice that did not receive CD4s.

In contrast to the effector CD4 subsets, I found that Tregs did appear to be negatively impacting on CD8 expansion/survival and tumour infiltration. This was despite the fact that the Tregs themselves had disappeared by day 7. In chapter 4, I found that adoptively transferred Tregs accumulate in abundance in the DLN and spleen at day 2 but do not survive until day 7. In addition, I did not find Tregs in the tumour, suggesting that the negative effect of Tregs on CD8 accumulation in the spleen and tumour seen at day 7 is likely to be a consequence of inhibitory activity in the DLN or spleen that occurs during the first few days after adoptive transfer, before the Tregs themselves have disappeared.

In addition there was no change in activation status or expression of PD-1 on HA specific CD8s in the presence of helper CD4 cells. PD-1 expression was highly upregulated on tumour infiltrating Thy1.1+ CD8s compared to that seen in the DLN or spleen, suggesting that the HA specific CD8s within the tumour are susceptible to negative regulation. Additionally PD-1 expression was higher on tumour infiltrating HA specific CD8s than on the endogenous CD8 cells, suggesting that this was not simply a global effect of the tumour microenvironment on all CD8s but was likely to be an effect of interactions between HA antigen and cognate CD8 cells. Ahmadzadeh et al found high levels of PD-1 expression on tumour infiltrating CD8s and this correlated with an exhausted phenotype and impaired effector function (Ahmadzadeh, Johnson et al. 2009). In viral models CD8 T cells primed in the absence of CD4 help were found to
excessively upregulate PD-1 (Fuse, Tsai et al. 2009) and this effect was also replicated by an absence of CD40-CD40L interactions (Dias, Giannoni et al. 2011). It is possible therefore that the HA specific CD8s within the tumour could have been functionally impaired as a result of ineffective CD4 help during priming.

Thus, the failure of CD4 helper subsets to provide help to CD8s in mice with established tumours could explain the lack of efficacy of the CD4 subsets in this setting. Potential reasons for this inability to help were considered:

1) Uncoupling of HA-HNT specificity.

Expression of HA on tumour cell lines was confirmed by others in the lab on frequent occasions by real time PCR (unpublished data - Amanda Cleaver). As reported in chapter 4, in vitro differentiated HNT CD4 cells retained the ability to respond to HA when restimulated with peptide in vitro and also expanded in vivo in the presence of HA expressing tumours, suggesting that loss of HA specificity on the adoptively transferred cells or loss of HA antigen expression on tumours was unlikely to be a factor. In addition work by others in the lab demonstrated that CFSE labelled HNT cells proliferated in vivo in the presence of AB1HA, demonstrating that host antigen presenting cells were able to process and present HA antigen (personal communication, Prof Richard Lake).

2) Overriding suppression from endogenous immunity.

Although we consistently saw survivors in Th1 treated mice in the prophylactic group, this was not replicated in established, gemcitabine treated tumours. It is noteworthy that following gemcitabine treatment at day 9, tumours rapidly regress to barely palpable by day 12 such that the overall tumour burden in the post gemcitabine
setting was not macroscopically dissimilar to that seen immediately following tumour inoculation. This suggests that there may be reasons other than increased tumour load to explain the differences in efficacy between the prophylactic and the treatment setting. Mice treated with gemcitabine at day 9 will have already established an immune response to tumour antigens, which is not the case in mice treated prophylactically. Daresse-Jeze et al showed that after first encounter with tumour antigens, regulatory T cells proliferate more rapidly than naive effector CD4 cells and suppress effector responses, but if effector memory T cells were present first then Tregs were unable to control responses (Darrasse-Jeze, Bergot et al. 2009). This suggests that the presence or absence of established regulatory cells at the time of adoptive transfer could determine outcome. Although gemcitabine does cause lymphodepletion, this is not complete, meaning that established regulatory T cells could still be suppressing the actions of adoptively transferred cells. Supporting this hypothesis was my finding that in established tumours, adoptively transferred Tregs had a negative impact on CD8 tumour infiltration, suggesting an environment which favoured the activity of suppressive subsets and not effector subsets. It was possible therefore that in the treatment model established endogenous Tregs were suppressing the activity of the adoptively transferred helper CD4 T cells.

3) Lack of ‘space’ for adoptively transferred cells to expand

In addition to depleting suppressive cells, lymphodepletion prior to adoptive transfer induces homeostatic proliferation and provides ‘space’ for adoptively transferred cells to expand (Weber, Atkins et al. 2011). Most adoptive transfer protocols employ some form of preconditioning to induce homeostatic proliferation of transferred cells. However, since Th1s were able to cause tumour rejection in the prophylactic model,
despite the absence of lymphodepletion, the requirement for ‘space’ cannot be considered necessary for their efficacy and does not in itself explain why these cells were ineffective when given after gemcitabine.

4) Lack of helper function on adoptively transferred CD4 subsets

Although Th1, Th2, Th17 and Treg cells are generally defined by expression of IFNγ, IL-4, IL-17 and foxp3 respectively, CD4 cells provide help to other immune cells by a number of mechanisms. Despite having the appearance of fully differentiated and functional CD4 helper cells, in vitro differentiated HA specific CD4 cells did not provide help to HA specific CD8s. Two of the most important mechanisms by which CD4 cells provide help are through activation of DCs through CD40-CD40L interactions and through production of IL-2 (Wilson and Livingstone 2008). It was therefore important to further define the phenotype of in vitro differentiated CD4 subsets and determine whether they were functionally able provide help to CD8 T cells through these mechanisms.

In summary the experiments described in this chapter show that only Th1 polarised CD4 cells have significant anti-tumour activity in vivo and that this is only in the prophylactic setting. The lack of effect against established tumours even after gemcitabine treatment could be explained by the apparent lack of help given to cognate CD8s. These results led me to investigate i) whether there were functional deficiencies in in vitro differentiated CD4 cells which limited the help they could provide and ii) whether removing the endogenous, suppressive CD4 repertoire prior to treatment would improve the capacity of adoptively transferred Th1s to eradicate established, gemcitabine treated tumours.
6 Investigating the ‘helper’ functions of in vitro differentiated CD4 cells
6.1 Introduction

The experiments described in this chapter follow from the discussion at the end of chapter five. The lack of activity of in vitro differentiated CD4 cells against established tumours, and the inability of these cells to mediate expansion and tumour infiltration of HA specific CD8 T cells in mice with established tumours, suggested the CD4 cells were failing to provide effective ‘help’ to HA specific CD8s.

I therefore examined the CD4 subsets for expression of markers associated with CD4 help, including CD40L (CD154) and IL-2. CD40L expressed on helper T cells ligates CD40 on dendritic cells, leading to dendritic cell activation and upregulation of co-stimulatory molecules and expression of IL-12 (Cella, Scheidegger et al. 1996; Schoenberger, Toes et al. 1998). IL-2 is a T cell growth factor which is important for sustaining CD8 T cell expansion, function and memory formation (Antony, Piccirillo et al. 2005; Janssen, Droin et al. 2005). A deficiency in either CD40L expression (Shah, West et al. 2009) or IL-2 production (Antony, Piccirillo et al. 2005) impairs the ability of CD4s to promote CD8 expansion or tissue infiltration.

I then tried to see if altering the culture conditions could alter the phenotype of the in vitro differentiated cells, with aim of improving their ability to provide help. Since Th1s were the only CD4 subset which had anti-tumour activity in the prophylactic setting, in these experiments I concentrated on this subtype. In some experiments I tried culturing Th1 cells in IL-7 rather than IL-2. IL-7 is a member of the common gamma chain family of cytokines and signals through the IL-7 receptor (CD127), which is expressed on naive T cells and on some effector T cells destined to become memory cells (Mackall, Fry et al. 2011). IL-7 was superior to IL-2 for the ex vivo expansion of
tumour specific T cells and produced cells with greater in vivo anti-tumour activity (Caserta, Alessi et al. 2010; Cha, Graham et al. 2010).

Once I had adjusted the culture conditions to produce Th1 cells which displayed a phenotype in vitro that might be associated with an improved ability to provide help to CD8 T cells, I adoptively transferred these cells into gemcitabine treated tumour bearing mice, and the effect on tumour growth and on the expansion and tumour infiltration of HA specific CD8s was analysed.
6.2 Results

6.2.1 In vitro differentiated helper T subsets express variable levels of CD40L, IL-2 and PD-1

Since the in vivo activity of in vitro differentiated CD4 T cells subsets had been weak and I had found no evidence that they had been able to help HA specific CD8s to expand or infiltrate tumours I looked to see whether these cells expressed markers associated with T cell help and also whether they may be susceptible to negative regulation. I examined expression of CD40L, IL-2 and PD-1 on the CD4 subsets at the end of the 5 day culture period (Figure 6-1).

CD40L expression was high on Th1 subsets, lower on Th0s and Th17s and minimally expressed on Th2s and Tregs. When cells were restimulated and analysed for expression of IL-2 by intracellular flow cytometry, surprisingly Th1s and Th2s produced no IL-2, whereas it was produced in abundance by Th17s, Tregs and Th0s. PD-1 was expressed most highly on Th17s with the lowest levels being seen on Tregs and Th0s, suggesting that Th17s had the greatest potential to be inhibited through ligation of PD-1.

These results indicated that an inability to provide help through CD40-CD40L interactions or through the production of IL-2 may have explained the lack of anti-tumour activity of the CD4 subsets. No cell type expressed both high levels of CD40L and IL-2. Loss of IL-2 expression was particularly surprising on the Th1 subset, since
this is often considered a Th1-type cytokine. I therefore went on to look at the kinetics of expression of CD40L and IL-2 during the whole of the 5 day culture period.

Figure 6-1 **Expression of CD40L (CD154), IL-2 and PD-1 on CD4 subsets at the end of 5 day in vitro culture.**

In vitro differentiated CD4 subsets were analysed for expression of CD40L and PD-1 by flow cytometry and are shown compared to the FMO control (unfilled histogram). IL-2 expression was analysed by intracellular flow cytometry after restimulation with PMA and ionomycin in the presence of brefeldin A. All plots gated on CD4+ cells. Plots representative of duplicate samples.
6.2.2  CD40L expression declines during in vitro culture in all CD4 subtypes except Th1

CD4 cells were differentiated in vitro into Th1, Th2, Th17, Treg and Th0s over 5 days in accordance to the protocols established in chapter 4. On days 0, 1, 2, 3 and 5 samples of cells were stained for expression of CD40L (Figure 6-2). This showed that CD40L was highly upregulated on Th1s, Th2s and Th0s after 24 hours activation but had declined in Th2s by day 3. Th0s lost CD40L between day 3 and day 5, whereas Th1s were the only subset to maintain CD40L throughout the 5 day culture. In contrast Th17s and Tregs expressed much lower levels after 24 hours of activation and this was lost by day 2 of culture.
Figure 6-2  Kinetics of CD40L expression during 5 day in vitro differentiation on CD4 subsets.

Expression of CD40L was determined by flow cytometry on day 0, 1, 2, 3 and 5 of in vitro differentiation. Gate set against FMO control.
I then looked to see if Th1s maintained expression of CD40L in vivo following adoptive transfer into gemcitabine treated tumour bearing mice. Expression of CD40L on adoptively transferred Th0s was also examined as a comparison. AB1-HA tumour bearing mice were injected with 5x10^5 in vitro differentiated HNT-Thy1.1 Th1s or Th0s and DLN and spleen were harvested 48 hours later. The frequency of CD40L+ Thy1.1+ve CD4 cells was determined by flow cytometry (Figure 6-3). This showed that although CD40L was expressed by a high proportion of Th1s before adoptive transfer, after two days in vivo less than 5% of cells recovered from the DLN or spleen still expressed CD40L and CD40L expression was now similar to that observed on Th0s, both in the DLN (p=0.11) and in the spleen (p=0.95).
Figure 6-3  Ex vivo expression of CD40L on adoptively transferred Th1s and Th0s 2 days after adoptive transfer.

Mice bearing AB1-HA tumours which had been treated with a single dose of 240ug/g gemcitabine on day 9 received adoptive transfer of $5 \times 10^5$ HNT Th1s or Th0s on day 11. DLN and spleen were harvested 48 hours after adoptive transfer and recovered cells analysed by flow cytometry. A Gating on CD4+Thy1.1+ cells and on CD40L+ (filled histogram) which are shown alongside FMO control (unfilled). B Proportion of CD40L+ cells amongst Thy1.1+CD4+ cells before adoptive transfer, and when recovered from the DLN and spleen 2 days after transfer (n=3 per group)
6.2.3  In vitro differentiated Th1s and Th2s lose IL-2 expression by day 3 of culture

Expression of IL-2 by in vitro differentiated CD4 cells was examined by intracellular flow cytometry on cells restimulated with PMA and ionomycin on day 0, 1, 2, 3 and 5 of culture. Comparison of IL-2 expression with expression of IFNg, IL-4, IL-17 and foxp3 on each respective subtype is shown in Figure 6-4. IL-2 expression is high 24 hours after activation in all subtypes, but in Th1s and Th2s it has disappeared by day 3. Interestingly this is at the point that expression of IFNg and IL-4 appears, such that there is only a short window where IFNg/IL-4 and IL-2 are both expressed. In Th17s, Tregs and Th0s, however, IL-2 expression is maintained throughout in vitro culture, indicating that the loss of IL-2 production by in vitro cultured Th1s and Th2s is specific to the Th1 and Th2 differentiation pathways.

Figure 6-4  Kinetics of IL-2 expression on in vitro differentiated CD4 subsets.

In vitro differentiated cells were restimulated with PMA and ionomycin and analysed by intracellular flow cytometry at each indicated timepoint. Expression of IL-2 alongside IFNg for Th1s and Th0s, IL-4 for Th2s, IL-17 for Th17s and foxp3 for Tregs is shown. Data is representative of two independent experiments for Th1, Th2 and Th0 subsets and 1 experiment for Th17s and Tregs. Missing data is due to failed restimulation at one of the timepoints in one experiment.
<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Th1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>6.45</td>
<td>11.03</td>
<td>40.19</td>
<td>33.84</td>
<td>63.14</td>
</tr>
<tr>
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<td>34.00</td>
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<td>3.97</td>
</tr>
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<td>0.66</td>
<td>8.62</td>
<td>1.63</td>
<td>6.23</td>
</tr>
<tr>
<td>Th17</td>
<td></td>
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</tr>
<tr>
<td>IL2</td>
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<td>72.14</td>
<td>27.02</td>
<td>0.99</td>
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</tr>
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<td>36.45</td>
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<td>44.66</td>
<td></td>
<td>53.92</td>
</tr>
<tr>
<td>Foxp3</td>
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<td>6.96</td>
<td>35.11</td>
<td>38.63</td>
<td>76.96</td>
</tr>
<tr>
<td>Th0</td>
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<td></td>
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</tr>
<tr>
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<td>32.44</td>
<td>82.09</td>
<td>73.09</td>
<td></td>
<td>48.99</td>
</tr>
<tr>
<td>IFNγ</td>
<td>6.45</td>
<td>5.13</td>
<td>3.54</td>
<td></td>
<td>1.75</td>
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</tbody>
</table>
I next examined whether the lack of IL-2 expression on in vitro differentiated Th1s persisted in vivo after adoptive transfer into tumour bearing mice. IL-2 and IFNγ expression on Thy1.1+ HNT Th1 cells recovered from AB1-HA tumour bearing mice, was determined by ex-vivo restimulation with PMA and ionomycin and intracellular flow cytometry (Figure 6-5A). After 7 days in vivo, around 40% of Th1 cells recovered from the spleen and tumour had regained the capacity to produce IL-2 but this was seen in only 10% of Th1 cells recovered from the DLN (Figure 6-5B).
Figure 6-5  Ex vivo IL-2 and IFNg expression on recovered Th1s 7 days after adoptive transfer.

AB1HA tumour bearing mice received adoptive transfer of $1 \times 10^6$ HNT Th1s. DLN, spleen and tumour were harvested 7 days later and cells restimulated ex vivo with PMA and ionomycin in the presence of brefeldin A and analysed by intracellular flow cytometry. A Gating on CD4+thy1.1+ cells. IFNg and IL-2 gates were set against FMO and unstimulated controls. B Expression of IFNg and IL-2 on recovered CD4+thy1.1+ve cells. (N=12 and shows pooled data of recovered thy1.1+CD4 cells from all treatment groups in experiment described in Figure 7-3.)
6.2.4 IFNg and IL-2 expression by in vitro differentiated Th1s resembles that seen in endogenous CD4s recovered from tumours but not the DLN

Although differentiating CD4 cells in vitro may not replicate what happens when CD4 cells differentiate in vivo, I was interested to see if there was any suggestion that CD4 cells primed in vivo also go through stages of differentiation involving differential expression of IL-2 and IFNg, similar to that seen in vitro. The in vitro data suggested that IL-2 is expressed in the early phases of Th1 differentiation and IFNg appears later, as IL-2 production is lost. I therefore re-examined the data obtained from the experiment described in Figure 5-7, where tumour bearing mice had received adoptive transfer of CD4 subsets, but gated on endogenous (thy1.1-ve) CD4 cells. IFNg and IL-2 expression on endogenous CD4 cells recovered from the DLN (the site of priming), the spleen and the tumour (the effector site) was analysed (Figure 6-6). This showed that CD4 cells in the DLN and spleen predominately produce IL-2, whereas CD4 cells in the effector site predominately produce IFNg. The proportion of IFNg+IL2+ double positive cells was lowest in the DLN but higher in the spleen and tumour.
Figure 6-6  IL-2 and IFNg expression on endogenous CD4+ cells in mice bearing AB1HA tumours.

Mice bearing AB1HA tumours received adoptive transfer of $1 \times 10^6$ thy1.1+HNT in vitro differentiated CD4 cells and $1 \times 10^6$ CL4 lymphocytes on day 9 after tumour inoculation and were culled on day 16, as described in Figure 5-7. Cells recovered from DLN, spleen and tumour were restimulated with PMA and ionomycin in the presence of brefeldin A and analysed by intracellular flow cytometry. IFNg and IL-2 expression on endogenous CD4+ (thy1.1-ve) cells was determined as shown. N=18.

Comparing these findings to what was seen on in vitro differentiated subsets it appeared that in vitro differentiated Th1 cells at the end of 5 days culture were more similar in phenotype to those endogenous CD4 cells found in the effector site than in the priming site, with high frequency of IFNg + cells and a low frequency of IL-2 producing cells.

Although IL-2 expression by adoptively transferred Th1s was regained in the spleen and tumour by day 7, this was not seen in the DLN. It was therefore possible that the
lack of help provided by in vitro differentiated CD4 cells to CD8s could be due to an inability to produce IL-2 in the DLN during CD8 priming.

6.2.5 Expanding ‘early’ activated Th1s in IL-7 maintains IL-2 and IFNg expression

Since IL-2 expression was progressively lost during the activation phase of Th1 cultures, I tried to see if I could preserve the IL-2 producing phenotype of ‘early’ Th1s by removing the activation stimulus after 24 hours. IL-2 and IL-7 are cytokines which have been used for in vitro expansion of tumour specific T cells and IL-7 produced cells with greater anti-tumour activity than IL-2 (Caserta, Alessi et al. 2010). I therefore tried expanding Th1 cells which had been activated for only 24 hours in either IL-2 or IL-7 for the remaining 4 days of culture in the presence of IL-12 but without anti CD3/CD28. For comparison, Th0s were treated in an equivalent way, without the addition of IL-12 to media (Figure 6-7A).
A HNT CD4 cells were activated with anti-CD3 and anti-CD28 for 24 hours under Th1 and Th0 polarising conditions. Cells were then washed and plated in fresh media containing IL-12 and anti-IL-4 for Th1s, or anti-IL4 and anti-IFNg for Th0s, in the absence of activation for a further 4 days. IL-2 at 50U/ml or IL-7 at 25ng/ml was added.

**Figure 6-7** The effects of expanding 1 day activated Th1s and Th0s in IL-2 and IL-7.
separately to individual wells after plating. Samples of cells were restimulated and analysed at day 1 and day 5 for expression of IFNg and IL-2 and unstimulated cells were analysed for expression of CD62L, CD40L and PD-1. Data shown is representative of two independent experiments. B Cells were treated as A, but after 24 hours activation Th1 cells were plated in media containing IL-12 and anti-IL4 only and analysed on day 5. C Cells were activated for 48 hours and 72 hours before being treated as described in A and analysed at day 5. B and C were performed once.

The phenotypes of Th1s and Th0s were similar at 24 hours, with high levels of IL-2 but little IFNg production. Th1 cells expanded for a further 4 days in IL-2 produced little IL-2 but contained a high proportion of IFNg secreting cells, similar to that seen previously when Th1 cells were activated for the full 3 days. In contrast, Th1 cells which were cultured in IL-7 following initial activation displayed slightly lower levels of IFNg expression, but produced large amounts of IL-2, with around 20% of cells positive for both IFNg and IL-2 (Figure 6-7A). The amount of proliferation was reduced in the IL-7 cultured cells, which expanded between 4.5 - 7 fold between day 1 and day 5, compared to a 16 fold expansion of the IL-2 cultured cells. This experiment was repeated comparing the effects of IL-7 on ‘early’ Th1s to the effects of simply resting the cells in media containing IL-12 and anti-IL4 but without any other cytokines (Figure 6-7B). This showed that IL-2 and IFNg production was similar in cells ‘rested’ for 4 days to cells cultured in IL-7, however these cultures produced only a 2 fold expansion of cells. Thus it appeared that culturing ‘early’ Th1 cells in IL-7 had the dual effect of preserving IL-2 production whilst expanding cells to sufficient numbers for adoptive transfer into mice.
In contrast, ‘early’ Th0s maintained similar levels of IL-2 production after expansion for 4 days in either IL-2 or IL-7 (Figure 6-7A). Again this indicated that the loss of IL-2 production by Th1s in IL-2 driven cultures was specific to the Th1 differentiation pathway. Th0s cultured in IL-2 expanded 21 fold between days 1 and 5, whereas those cells cultured in IL-7 expanded 4.1 fold.

Removing the activating stimuli from Th1s after 48 and 72 hours and then culturing them in IL-2 or IL-7, induced cells that produced little IL-2 at the end of 5 days culture (Figure 6-7C) irrespective of whether IL-2 or IL-7 was added to the culture, indicating that the ability to preserve the ‘early’ IL-2 producing phenotype of Th1s does not persist once cells have been activated for longer than 24 hours.

Examination of other phenotypic markers showed that expression of CD62L, CD40L and PD-1 was similar between IL-7 and IL-2 expanded ‘early’ Th1s and Th0s, with very high levels of CD62L and low levels of CD40L and PD-1 (Figure 6-7A). Thus Th1 cells activated for only 24 hours and then cultured with IL-7 and IL-12 display a phenotype which suggests that they may have the potential to traffic to lymph nodes in vivo and produce both IL-2 and IFNγ. Expression of IFNγ, IL-2, CD40L and CD62L on Th1s activated for 24 and 72 hours and on Th1s activated for 24 hours and expanded in IL-7, is summarised in Table 6.1.

Since expanding 1 day activated Th1s in IL-7 preserved the high levels of IL-2 production seen in the early stages of Th1 differentiation, I labelled these cells ‘early Th1s’, whereas cells activated for 3 days and expanded in IL-2 are referred to as ‘late Th1s’.
### Table 6-1  Summary of characteristics of Th1 cells activated for 24 hours and expanded in IL-7 or activated for 72 hours and expanded in IL-2.

<table>
<thead>
<tr>
<th></th>
<th>Naive CD4 cells</th>
<th>After 24 hours in vitro activation</th>
<th>Activated 24 hours IL-7 expanded day 1-5 ‘Early Th1s’</th>
<th>Activated 72 hours IL-2 expanded day 3-5 ‘Late Th1s’</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNg</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>IL-2</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>CD40L</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CD62L</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+</td>
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</tbody>
</table>
6.2.6 1 day activated, IL-7 expanded ‘early’ Th1s home to lymph nodes and produce IL-2

I next examined the behaviour of 1 day activated, IL-7 expanded ‘early’ Th1s in vivo, to determine if the adjustment in polarising conditions had altered their behaviour in vivo following adoptive transfer.

Mice bearing 14 day old AB1-HA tumours were treated with a single dose of 240μg/g gemcitabine and 48 hours later received adoptive transfer of 5x10^5 1 day activated, IL-7 expanded ‘early’ Th1s, 3 day activated IL-2 expanded ‘late’ Th1s or Th0s. An equal number of thy1.1+ CL4 CD8 T cells were transferred at the same time. Larger tumours were used so that at the time of harvest tumours would still be of sufficient size to yield adequate numbers of cells for analysis. DLN, spleen and tumour were harvested 2 days and 7 days and cells analysed by flow cytometry (Figure 6-8).

Analysis of recovered thy1.1+ve CD4 cells revealed that ‘early’ Th1s and ‘late’ Th1 are found in equal proportions in the spleen at day 2 following adoptive transfer. However, consistent with their high CD62L expression, ‘early’ Th1s accumulate in greater quantities in the DLN at day 2 (p<0.05) (Figure 6-8A), suggesting that ‘early’ Th1s may be quantitatively more able to provide help to HA specific CD8s in the DLN than ‘late’ Th1s. Th0s did not survive well after adoptive transfer and few cells were recovered at day 2.
Figure 6-8  Tracking of ‘late’ Th1s, ‘early’ Th1s and Th0s following adoptive transfer into tumour bearing mice treated with gemcitabine.

Mice bearing 14 day old AB1-HA tumours were treated with 240μg/g gemcitabine. 48 hours later mice received transfer of 5x10⁵ thy1.1+HNT ‘late’ Th1s, ‘early’ Th1s or Th0s with 5x10⁵ thy1.1+CD48 T cells. At 2 and 7 days after adoptive transfer mice were culled and cells recovered from DLN, spleen and tumour were analysed by flow cytometry (3 mice per group). Cells were gated on CD4+Thy1.1+ cells as per figure 4.5. Cell counts were determined as described in materials and methods. A  Recovery of
Thy1.1+CD4+ cells as a percentage of total CD4 cells.  B IFNγ and IL-2 expression on Thy1.1+CD4+ cells recovered 2 days after adoptive transfer and from the DLN and spleen and restimulated with PMA and ionomycin and analysed by intracellular flow cytometry.  C Frequency of Thy1.1+CD4+ cells in tumour at day 7 expressed as percentage of total CD4 cells and also as percentage of total cells, gated from forward and side scatter plots as illustrated in figure 5-7.  Experiment performed once, 3 mice per group. * denotes significance at p<0.05.

The phenotype of the cells was examined 2 days after adoptive transfer, cells were restimulated with PMA and ionomycin and expression of IFNγ and IL-2 was characterised by flow cytometry (Figure 6-8B).  This showed that after 2 days in vivo, the adoptively transferred cells retained the phenotype they possessed at the end of in vitro culture.  A high proportion of 3 day activated, ‘late’ Th1s expressed IFNγ but few produced IL-2; Th0s made IL-2 but did not express IFNγ and 1 day activated, ‘early’ Th1s expressed high levels of both IFNγ and IL-2.

Tumour infiltrating thy1.1 CD4s were not detected 2 days after adoptive transfer, although tumour sizes were small (10-20mm²) at this point.  However by 7 days after adoptive transfer, tumour infiltrating thy1.1+ve CD4s could be detected.  This showed that only 3 day activated, ‘late’ Th1s infiltrated tumours in any great quantity at this time point, where they comprised 1 -6 % of the total CD4 population (Figure 6-8C), although did not reach significance compared to ‘early’ Th1s (p=0.10).

Thus ‘early’ (IL-7 expanded) Th1s preferentially accumulate in the DLN, where they are able to produce IL-2, but they do not infiltrate the tumour.  ‘Late’ Th1s home to the tumour and are able to produce IFNγ.  ‘Late’ Th1s which do make it into the DLN are unable to produce IL-2.
6.2.7 The post gemcitabine environment does not favour survival of in vitro differentiated Th1s beyond day 2 after adoptive transfer

Unexpectedly, between day 2 and 7 numbers of both ‘late’ and ‘early’ Th1s declined in the spleen and ‘early’ Th1s also declined in the DLN (Figure 6-8A). This is in contrast to results presented in section 4.2.7, where a 3 fold expansion of Th1s in untreated tumour bearing mice was observed during the same period. Since gemcitabine was not directly killing the adoptively transferred cells, the differences observed between untreated and gemcitabine treated mice indicate that the post gemcitabine environment did not favour the expansion of the transferred HA specific cells.

In chapter 5 it was demonstrated that cells adoptively transferred 24 hours or more after gemcitabine were not killed by the gemcitabine. Failure of the adoptively transferred cells to expand in the post gemcitabine setting suggested that these cells did not undergo either antigen driven proliferation or homeostatic proliferation, as seen in the endogenous lymphocyte population following gemcitabine (see Figure 3-1A). This could have been due to reduced ability to respond to cytokines such as IL-7 or IL-2, or reduced availability of these cytokines. I therefore compared expression of CD25 (IL-2 receptor) and CD127 (IL-7 receptor) on ‘early’ Th1s, ‘late’ Th1s and naive (CD62L+) CD4 cells (Figure 6-9).

Naive cells express high levels of the IL-7 receptor, but undetectable levels of the IL-2 receptor. ‘Late’ Th1s expressed very high levels of the IL-2 receptor but lower levels of IL-7 receptor. ‘Early’ Th1s expressed very low levels of the IL-7 receptor and although CD25 was detectable in the majority of ‘early’ Th1s, this was mostly at an intermediate rather than high level of expression. Thus during a period of homeostatic, IL-7 driven,
proliferation, in vitro differentiated cells may be at a disadvantage to naive endogenous cells due to lower levels of expression of the IL-7 receptor, which may explain the reduced expansion and survival observed.

Figure 6-9  CD25 and CD127 expression on naive CD4 cells, ‘early’ Th1s and ‘late’ Th1s.

Naive CD4+CD62L+ cells were isolated by magnetic column separation of HNT splenocytes. ‘Early’ and ‘late’ Th1s were generated over 5 days in vitro, as described in Figure 6-7. CD25 and CD127 expression was determined by flow cytometry. Plots show CD25 and CD127 expression (filled histograms) and FMO control (unfilled). Performed once.
6.2.8 1 day activated ‘early’ Th1s do not improve outcomes when transferred after gemcitabine

I hypothesised that the inability of in vitro differentiated Th1s to produce IL-2 in the DLN could explain the lack of help provided to CD8s and the lack of efficacy of these cells against established tumours. I therefore compared the anti-tumour activity of 3 day activated, IL-2 expanded Th1s (‘late’ Th1s), which do not make IL-2, with 1 day activated, IL-7 expanded Th1s (‘early’ Th1s), which make IL-2, as well as with Th0s (make IL-2). Additionally, since it was possible that optimal anti-tumour responses might require both ‘early’ IL-2 producing Th1s in the DLN and ‘late’ IFNg producing Th1s at the effector site, I also looked at the effects of transferring ‘late’ Th1s mixed at a 1:1 ratio with either ‘early’ Th1s or Th0s. As before, all groups additionally received adoptive transfer of HA specific CD8s cells.

Mice bearing 9 day old AB1-HA tumours were treated with a single dose of gemcitabine followed 48 hours later by adoptive transfer of $5 \times 10^5$ CL4 CD8 T cells in conjunction with $5 \times 10^5$ in vitro differentiated thy1.1 HNT CD4s. The CD4s consisted of either ‘late’ Th1s, ‘early’ Th1s, Th0s, ‘late’ Th1s plus ‘early’ Th1s, or ‘late’ Th1s plus Th0s.

There was no significant difference in tumour growth or survival between any of the groups (figure 6-10). No long term survivors were seen.
Mice bearing 9 day old AB1-HA tumours were treated with 240μg/g gemcitabine i.p. and 48 hours later received intravenous injection of 5x10^5 CL4 CD8 T cells alone or of 5x10^5 CL4 CD8 T cells with 5x10^5 in vitro differentiated HNT CD4 cells - consisting of ‘late’ Th1s, ‘early’ Th1s, Th0s, ‘late’ Th1s mixed 1:1 with Th0s or ‘late’ Th1s mixed 1:1 ‘early’ Th1s. 6 mice per group. Experiment performed once.

Figure 6-10  Effects of ‘early’ and ‘late’ Th1s on ability of mice to reject tumour following gemcitabine
The lack of effect of any of the CD4 subsets on tumour growth is reflected in a lack of positive effects of any of the CD4 combinations on the recovery of HA specific CD8s. Recovery of thy1.1+ CD8s from the DLN and spleen was not altered by co-transfer of any of the CD4 subtypes (Figure 6-11A). Thy1.1 CD8 cells were detectable in the tumour at day 7, however again the presence of any of the different types or combinations of CD4 cell did not increase HA specific CD8 tumour infiltration compared to mice that received CL4 CD8 cells alone (Figure 6-11B).
A  

**Figure 4.12**

**A**

**DLN Thy1.1 CD8s**

<table>
<thead>
<tr>
<th>Group</th>
<th>% Thy1.1+ve/CD8s</th>
</tr>
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<tbody>
<tr>
<td>'late' Th1</td>
<td>0.5</td>
</tr>
<tr>
<td>'early' Th1</td>
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</tr>
<tr>
<td>Th0</td>
<td>0.0</td>
</tr>
<tr>
<td>'late' Th1 + Th0</td>
<td>0.0</td>
</tr>
<tr>
<td>'late' Th1 + 'early' Th1</td>
<td>0.0</td>
</tr>
<tr>
<td>CL4s only</td>
<td>0.0</td>
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**Spleen Thy1.1 CD8s**

<table>
<thead>
<tr>
<th>Group</th>
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<tbody>
<tr>
<td>'late' Th1</td>
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<tr>
<td>'early' Th1</td>
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<tr>
<td>Th0</td>
<td>0.0</td>
</tr>
<tr>
<td>'late' Th1 + Th0</td>
<td>0.0</td>
</tr>
<tr>
<td>'late' Th1 + 'early' Th1</td>
<td>0.0</td>
</tr>
<tr>
<td>CL4s only</td>
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B

**Tumour Thy1.1 CD8s**

<table>
<thead>
<tr>
<th>Group</th>
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<tbody>
<tr>
<td>'late' Th1</td>
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<tr>
<td>'early' Th1</td>
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<tr>
<td>Th0</td>
<td>0.0</td>
</tr>
<tr>
<td>'late' Th1 + Th0</td>
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</tr>
<tr>
<td>'late' Th1 + 'early' Th1</td>
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<tr>
<td>CL4s only</td>
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**Tumour Thy1.1 CD8s**

<table>
<thead>
<tr>
<th>Group</th>
<th>% Thy1.1+ve/CD8s</th>
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<td>Th0</td>
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<tr>
<td>CL4s only</td>
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**Figure 6-11**  Tracking of CL4 Thy1.1+CD8s injected alongside HNT CD4 cells.

Mice were treated as described in Figure 6-10, receiving $5 \times 10^5$ CL4 thy1.1+CD8s along with $5 \times 10^5$ in vitro differentiated HNT ‘late’ Th1s, ‘early’ Th1s, Th0s, or no HNT CD4s. In addition, two further groups of mice received $5 \times 10^5$ CL4 Thy1.1+CD8s alongside $2.5 \times 10^5$ ‘late’ Th1s mixed with either $2.5 \times 10^5$ ‘early’ Th1s or with Th0s. Harvests on mixed groups were only performed at day 7. **A** Recovery of thy1.1+CD8+ cells from DLN and spleen expressed as percentage of total CD8s. **B** Recovery of thy1.1+CD8+
cells from the tumour 7 days after adoptive transfer, expressed both as a percentage of total CD8s and of total cells.
6.3 Discussion

In this chapter I examined the expression of two important mediators of CD4 T cell help on in vitro differentiated CD4 subsets - CD40L and IL-2. I found that on 5 day in vitro differentiated cells, CD40L was only expressed at high levels on Th1s. This could potentially have explained the anti-tumour activity of Th1s seen in the prophylactic situation and the absence of activity of other subsets. When I examined the kinetics of CD40L expression during the 5 day period of activation and expansion of CD4 subsets I found that Th1s, Th2s and Th0s initially express high levels of CD40L whereas Th17s and Tregs express lower amounts, suggesting that expression of CD40L following initial activation is not consistent between different CD4 differentiation pathways.

Furthermore only Th1s maintained high levels of CD40L expression until day 5 of culture. Lee et al compared CD40L on CD4s activated in Th1 (IL-12) and Th2 (IL-4) cultures and similarly found that both conditions induced high initial CD40L expression but it was only in the presence of Il-12 that expression was sustained for extended periods (Lee, Haynes et al. 2002).

Thus, expression of CD40L is dynamic during in vitro culture of CD4 cells and depends on the polarising cytokines added to cultures. As a consequence the ability to provide help through CD40 activation in vivo could potentially differ between subsets.

However when I examined cells recovered from mice two days after adoptive transfer, CD40L was downregulated on Th1s and Th0s in cells recovered from the DLN and spleen. Together these results suggested that CD40L expression is not maintained after initial activation unless IL-12 is present and that expression of CD40L during in vitro culture is not necessarily retained in vivo after adoptive transfer. It is unclear
from these results whether in vitro differentiated CD4 subsets can re-express CD40L after meeting antigen in vivo and what role that local concentrations of cytokines such as IL-12 in vivo might have on CD40L expression on adoptively transferred CD4 cells.

Surprisingly, in vitro differentiated Th1s and Th2s do not make IL-2 at the end of the five day culture period, whereas the other subsets produced substantial quantities. IL-2 production was lost in Th1s and Th2s after day 2 of activation, around the time that IFNg and IL-4 started to be produced. The ability of IL-12 and IL-4 to suppress IL-2 production on in vitro activated T cells has been described (Dickensheets, Freeman et al. 2000; Villarino, Tato et al. 2007) and loss of IL-2 occurred by 48 hours (Villarino, Stumhofer et al. 2006). Interactions between Tbet and the RelA NFkB transcription factor link the Th1 differentiation pathway and loss of IL-2 production (Hwang, Hong et al. 2005). The fact that Th0 cells, which were treated identically but did not receive IL-12 or IL-4, sustained IL-2 production suggests that loss of IL-2 is a consequence of Th1/Th2 differentiation rather than simply a result of direct negative feedback from IL-2 itself. This is in contrast to the findings of Villarino et al who showed that IL-2 did negatively regulate IL-2 production (Villarino, Tato et al. 2007), although this could in part have been an indirect, IL-4 mediated effect as IL-2 itself can promote Th2 differentiation and hence IL-4 production (Zhu, Cote-Sierra et al. 2003).

Th1 cells recovered ex vivo 2 days after adoptive transfer did not produce IL-2 following restimulation. However this capacity had been regained by day 7 in a proportion of Th1s recovered from the spleen and tumour. Similarly, Villarino demonstrated that loss of IL-2 production on helper CD4s was not permanent as cells had regained the capacity to make IL-2 after 15 days in vivo (Villarino, Tato et al. 2007). However, in this model Th1 cells in the DLN still made little IL-2 7 days after transfer,
suggested that their helper function may still have been impaired in the priming phase.

The importance of IL-2 produced by CD4 T cells in promoting CD8 responses is well recognised. CD4 T cell derived IL-2 was found to be essential for supporting the expansion and survival of cognate CD8s (Antony, Piccirillo et al. 2005; Wilson and Livingstone 2008). CD8s which have been helped during priming by the provision of IL-2 are able to proliferate on secondary contact with antigen whereas ‘helpless’ CD8s undergo activation induced cell death (Janssen, Droin et al. 2005; Williams, Tyznik et al. 2006). However, a recent report suggests that autocrine IL-2 production by CD8 cells themselves determines CD8 memory formation, rather than paracrine production of IL-2 by helper CD4 cells (Feau, Arens et al. 2011). In tumour bearing mice CD4s were also found to provide help at the effector site, where secretion of IL-2 by CD4s induced increased proliferation of intratumoural CD8s, which demonstrated enhanced cytolytic capacity (Bos and Sherman 2010).

Studies which have generated CD4 subsets in vitro for use against tumours have often not reported IL-2 expression on the differentiated cells (Nishimura, Iwakabe et al. 1999; Martin-Orozco, Muranski et al. 2009). My results suggest that the ability of in vitro differentiated CD4 cells to produce IL-2 is highly dependent on the culture conditions employed, including the polarising cytokines, duration of activation stimulus and the addition of exogenous IL-2. In accordance with my findings, IL-2 production by cells stimulated with anti CD3 and anti CD28 was reduced when cells were polarised to Th1s (Dickensheets, Freeman et al. 2000). Additionally, studies that used peptide pulsed APCs to activate T cells report variability in IL-2 production by in vitro differentiated Th1s: Muranski found that peptide-activated tumour antigen specific
Th1s produced substantially less IL-2 than CD4s polarised to a Th17 phenotype (Muranski, Boni et al. 2008). Hegazy et al found that 4 days of Th1 culture with peptide activation produced over 60% IFNγ +ve cells but only around 10% IL-2 +ve cells (Hegazy and Klein 2008). However, Huang et al found IL-2 levels in the supernatant of peptide activated Th1s were only slightly lower than the levels of IFNγ (Huang, Hao et al. 2007). Rogers et al found that IL-2 was produced by CD4 T cells stimulated with low concentrations of peptide but was reduced when cells were stimulated with high concentrations of peptide, which may explain how both the nature and the duration of the activating stimuli could influence the ability of in vitro differentiated CD4s to produce IL-2 (Rogers and Croft 1999).

The lack of production of IL-2 on in vitro differentiated Th1s could have explained their inability to provide help to CD8s in mice bearing established tumours. I therefore looked to see whether I could alter the culture conditions in order to retain IL-2 production by the in vitro differentiated Th1s. It was possible to generate Th1 cells that produced both IFNγ and IL-2 if activation stimuli were removed after 24 hours and cells were cultured in IL-12 and IL-7 for the remainder of the 5 days. This phenotype was retained when cells were recovered after 2 days in vivo. Interestingly, culturing cells in IL-2 after 24 hours activation produced an IL-2 negative phenotype, similar to cells which had been activated for the full three days. This demonstrated that either ongoing TCR stimulation or expansion with IL-2 promotes loss of IL-2 production by in vitro differentiated, 1 day activated Th1s. 1 day activated Th1s which were ‘rested’ in media containing IL-12 without any other cytokines also retained the ability to produce IL-2, suggesting that it is the removal of TCR stimulation and IL-2 which is important, rather than the addition of IL-7 per se. However cells cultured in IL-7 expanded 2-3 fold more than rested cells, so the role of IL-7 in these cultures may have been to
promote expansion/survival of cells without switching off IL-2. Villarino et al found that IL-7, but not IL-15, partially inhibited IL-2 expression on CD4 cells but that addition of neutralising anti-IL-2 antibody to cultures abrogated this effect, suggesting IL-7 can inhibit IL-2 production but only in the presence of IL-2 itself (Villarino, Tato et al. 2007). Thus, using a neutralising antibody to remove any IL-2 still being produced by the activated CD4 cells could be another way to promote IL-2 production by helper T cells expanded in IL-7.

These ‘early’ activated, IL-7 expanded Th1s also expressed very high levels of CD62L, suggesting that they may have the potential to traffic to lymph nodes and provide IL-2 during CD8 priming. This was indeed shown to be the case, as 2 days after adoptive transfer they were found in the DLN at a frequency 6 fold greater than 3 day activated ‘late’ Th1s. Thus, expanding ‘early’ Th1s in IL-7 restores a CD62Lhi, lymph node homing phenotype, whereas ‘late’ Th1s accumulate slowly in the DLN. The importance of CD4 help in the early stages of CD8 priming was demonstrated by Lai et al, who showed that IL-2 provided by CD4 cells in the first two hours during in vitro stimulation of CD8 T cells promoted accumulation of viable CD8s, but this declined if IL-2 delivery was delayed by more than 3 hours (Lai, Lin et al. 2009). Therefore, following adoptive transfer, discordant initial trafficking to the DLN of the naive HA-specific CD8s and the helper CD4 cells could have explained the inability of ‘late’ Th1s to provide help during CD8 priming.

In contrast, ‘late’ Th1s may have a greater propensity to infiltrate tumours than ‘early’ Th1s, although this did not reach significance. I hypothesised therefore that ‘early’ Th1s may have a greater potential to provide help at the site of priming and ‘late’ Th1s may have the greater potential for activity at the effector site. Villarino et al proposed
a model whereby naive CD4 cells are first activated in the DLN, where they mainly produce IL-2, and then as they fully differentiate into Th1s they migrate to effector sites, by which time they become mainly IFNg secreting (Villarino, Stumhofer et al. 2006). In keeping with this, I also observed differential levels of IL-2 and IFNg secretion on endogenous CD4 cells recovered from the DLN and tumour. Additionally, the differing kinetics of IL-2 and IFNg production observed during in vitro differentiation of Th1s would also be consistent with theory that Th1 polarised CD4 cells may have different functions at different stages of differentiation. This raised the possibility that a complete Th1 CD4 helper response could require cells in multiple stages of differentiation, with both ‘early’ IL-2 secreting cells in the DLN and ‘late’ IFNg secreting cells at the effector site.

Overall the results described so far in this chapter again emphasise that the phenotype and in vivo behaviour of in vitro differentiated CD4 cells are highly dependent on the culture conditions used to produce them. Although 3 day activated, IL-2 expanded Th1s and 1 day activated, IL-7 expanded Th1s appeared similar in terms of IFNg production, their ability to produce IL-2 and to traffic to the DLN differed markedly.

Having adjusted the culture conditions such that I could generate Th1 cells which were able to secrete IL-2 as well as IFNg and were able to traffic to the DLN, I examined the anti-tumour activity of these cells and their ability to help HA specific CD8s in tumour bearing mice treated with gemcitabine. I also looked to see whether combining both ‘early’ and ‘late’ Th1s would improve outcomes. However, the addition of either of the types of Th1 did not have any positive effects on tumour rejection or on expansion of HA specific CD8s.
The reasons for this lack of efficacy could be explained by the unexpected failure of either the adoptively transferred HNT CD4 cells or the CL4 CD8 cells to expand to any great degree between day 2 and day 7. Indeed, there was a substantial decline in numbers of Th1s during this period. This was despite the cells being injected during the period of lymphodepletion induced by gemcitabine and a presumed increased availability of tumour antigens due to gemcitabine induced tumour cell death. Furthermore, this is the opposite to that observed in chapter 4, where Th1 cells administered to untreated tumour bearing mice expanded in all compartments between days 2 and 7.

The second hypothesis stated at the start of this thesis was that the post-gemcitabine environment would provide favourable conditions for adoptive transfer. The results in this chapter disprove this hypothesis. In actual fact the post gemcitabine environment appears to adversely affect the survival of adoptively transferred cells when compared to mice that did not receive gemcitabine. This is not due to direct cytotoxicity of gemcitabine on the transferred cells, but rather due to a failure of these cells to survive/expand between day 2 and 7 following transfer.

Experiments described in chapter 3 show that gemcitabine causes lymphodepletion followed by restoration of cell numbers by day 4. In addition I showed in chapter 3 that gemcitabine induces an endogenous immune response which may mediate tumour regression 7-10 days later. It therefore is likely that the adoptively transferred cells are in competition with proliferating endogenous immune cells following gemcitabine. Limited availability of growth factors such as IL-7 or IL-2 in this environment could explain the lack of proliferation of the adoptively transferred cells in the post gemcitabine setting compared to the untreated setting. Notably,
expression of the IL-2 receptor was lower on ‘early’ Th1s than ‘late’ Th1s and expression of the IL-7 receptor by both types of Th1 was substantially lower than that seen on naive cells, potentially putting the adoptively transferred cells at a disadvantage in such a scenario.

In summary the results described in this chapter demonstrate:

- Expression of helper functions, CD40L and IL-2, is dynamic during in vitro differentiation and varies between different CD4 subsets. IL-2 production by Th1s is highly dependent on the culture conditions, including duration of TCR activation and the addition of IL-2.
- My initial in vitro differentiation protocols generate ‘Th1’ cells which are able to infiltrate the tumour and produce IFNγ but are less able to track to the DLN, where they are unable to make IL-2.
- Expanding ‘early’ Th1s in IL-7 restores their ability to track to the DLN and produce IL-2, but this did not improve anti-tumour activity in the post gemcitabine setting.
- The post gemcitabine environment is not favourable for adoptive transfer of in vitro differentiated CD4 subsets. This is not due to direct cytotoxicity but due to failure of cells to expand between day 2 and 7 after transfer.
Removal of endogenous suppressive CD4 cells prior to treatment with gemcitabine and adoptive cell transfer
7.1 Introduction

Results in chapter 5 showed that adoptive transfer of HA specific Tregs reduced tumour infiltration of HA specific CD8s, whereas transfer of effector CD4 subsets had no impact on CD8 recovery. This suggested that the tumour environment was permitting the activity of suppressive CD4 cells but may be suppressing the activity of effector cells. Thus it was possible that overriding suppression from endogenous regulatory cells could have explained the lack of activity of adoptively transferred effector CD4 cells against established tumours, and hence the lack of help provided to cognate CD8s. In chapter 3 I showed that gemcitabine causes lymphodepletion and a reduction in tumour infiltrating Tregs 7 days after treatment, however depletion of Tregs was not complete and was not seen in the DLN, the site of CD8 priming. In addition, Tregs expand rapidly during the early phases of immune constitution following lymphopenia (Zhang, Chua et al. 2005; Rezvani, Mielke et al. 2006) meaning a more complete depletion of Tregs may be required to abrogate their suppressive effects. Additionally results in chapter 6 suggested that in the post gemcitabine environment adoptively transferred cells did not expand between day 2 and day 7, unlike that seen in untreated mice. One explanation for this could have been that the adoptively transferred cells were in competition with endogenous lymphocytes proliferating in response to gemcitabine induced lymphodepletion.

For these reasons, I hypothesised that removing endogenous CD4 cells prior to gemcitabine treatment and adoptive transfer would improve the ability of the transferred cells to reject tumours. Most adoptive transfer protocols in humans and mice have included a lymphodepleting preconditioning regimen, consisting of total
body irradiation, or highly myelosuppressive chemotherapy (Dudley, Wunderlich et al. 2002; Muranski, Boni et al. 2008). The intensity of the conditioning radiation correlated with efficacy of adoptive cell therapy (Wrzesinski, Paulos et al. 2011). However, these regimens deplete other immune cells as well as CD4 cells and are likely to have additional direct effects on the tumour, making it more difficult to determine the precise role CD4 cells play in outcomes. I therefore developed a technique by which endogenous CD4 cells could be depleted in vivo using a CD4 depleting antibody without adversely affecting the cells adoptively transferred one day later. This allowed me to examine whether the presence of endogenous, suppressive CD4 cells were limiting the effectiveness of adoptively transferred helper CD4s.

Since Th1s were the only subtype to have demonstrated any anti-tumour activity in any setting, all experiments in this chapter were performed using in vitro differentiated Th1s. As in previous experiments, these were co-transferred with HA specific naive CD8 T cells harvested from a naive CL4 mouse.
7.2 Results

7.2.1 Titrating the dose of depleting anti-CD4 antibody

In order to see whether suppressive endogenous CD4s were limiting the effectiveness of the adoptively transferred HA specific T cells against established, gemcitabine treated tumours, CD4 cells needed to be removed from mice prior to treatment. In vivo depletions of CD4 cells can be performed by injection of anti-CD4 antibody (GK1.5), with doses reported between 100ug to 500ug (Nowak, Robinson et al. 2003; Teng, Swann et al. 2010). One study showed that after high doses of GK1.5 CD4 cells remain depleted for over a week (Ghobrial, Boublik et al. 1989). I initially tried removing CD4 cells with 200ug of GK1.5 but I found that, although this produced 100% CD4 depletion, it also depleted thy1.1+ve CD4 cells adoptively transferred 3 days later (data not shown), indicating excess antibody was still active in vivo. Even when the dose of antibody was reduced to 25ug, thy1.1+ve CD4 cells transferred on day 3 after GK1.5 could not be recovered from spleens on day 6 after GK 1.5, even though the endogenous CD4 cells had started to regenerate by this time (data not shown). This suggested that once a saturating dose of anti-CD4 antibody had been administered, the window for adoptive transfer, in which anti-CD4 was no longer active in vivo but before the endogenous CD4 cells had returned, was very narrow. I therefore titrated down the dose of antibody further, aiming for a sub-saturating dose, such that the majority of endogenous CD4 cells would be depleted, but that there would be no excess antibody left to deplete any cells adoptively transferred 1 day later (Figure 7-1A).
When the dose of GK1.5 was reduced to 4ug, over 90% of CD4 cells were depleted from the peripheral blood 24 hours later (Figure 7-1B). At this dose, when harvests were performed four days after GK1.5, numbers of CD4 cells in the spleen were still depleted to around a third of baseline levels, whereas they were still 100% depleted with doses over 20ug (Figure 7-1C). Although I did not perform counts on lymph nodes at this time point, at day +4 the CD4:CD8 ratio was similar in the LN and spleen, demonstrating that CD4s were being depleted to an equivalent extent in the spleen and lymph nodes (Figure 7-1D). Importantly, recovery of thy1.1+ve CD4 cells adoptively transferred 1 day after GK1.5 was unaffected by doses up to 5ug, but these cells were completely depleted by doses over 10mg. This indicated that the saturation point lay between 5 and 10ug, above which excess antibody was still present to deplete CD4 cells transferred 24 hours later (Figure 7-1E). I therefore opted to use a dose of 4ug for experiments described in this chapter, where depletion of endogenous CD4 cells prior to adoptive transfer was required.

Figure 7-1   Titrating the dose of anti-CD4 depleting antibody.

A  Thy1.1- balb/c mice were injected intravenously with GK1.5 on day 0 and tail bleeds were performed 24 hours later to determine the depth of CD4 depletion. Mice then received adoptive transfer of 1.5x10^6 unmanipulated lymphocytes from a naive thy1.1+ve HNT mouse. 3 days after adoptive transfer spleens and lymph nodes were harvested and enumeration of recovered thy1.1+ve and thy1.1- CD4 cells was performed by flow cytometry. B  shows the degree of CD4 depletion in peripheral blood 1 day after GK1.5.  C shows numbers of splenic CD4 cells present at the time of harvest on day +4. D  shows the CD4:CD8 ratio in endogenous (thy1.1-) cells in the DLN and spleen on day +4. E  shows the number of adoptively transferred thy1.1+ve CD4 cells recovered from spleen at day +4 and the thy1.1+ CD4:CD8 ratio.
Figure 5.1    Titrating dose of CD4 depleting antibody

CD4s old + new batch combined

0 ug 1 ug 3 ug 4 ug 5 ug 7 ug 9 ug 10 ug 20 ug 50 ug 75 ug 100 ug

CD4s per ml

Endogenous CD4

0 ug 3 ug 4 ug 5 ug 7 ug 9 ug 20 ug 50 ug 75 ug 100 ug

0 5.0 \times 10^5 1.0 \times 10^6 1.5 \times 10^6 2.0 \times 10^6 2.5 \times 10^6 3.0 \times 10^6

CD4s per spleen

Dose anti-CD4

Blood 1 day post aCD4

Spleen 4 days post aCD4

Endogenous CD4:CD8 ratio day +4 spleen

Endogenous CD4:CD8 ratio day +4 DLN

Recovered Thy1.1+ve CD4s day +4 spleen

Thy1.1+ve CD4:CD8 ratio day +4 spleen

A

Gated on CD3+

Day 0 +1 +4
Bleed Harvest

B

C

D

E

Thy1.1 CD4s
cells

Gated on CD3+

Thy1.1 CD4:CD8

Recovered Thy1.1+ve CD4s day +4 spleen

Thy1.1+ve CD4:CD8 ratio day +4 spleen

Cells per spleen

Dose anti CD4

Dose anti CD4

Dose anti CD4

Dose anti CD4
7.2.2 Depletion of endogenous CD4 cells prior to adoptive transfer of HA
specific Th1s and CD8s improves survival in the post gemcitabine
treatment setting

To test whether removal of endogenous CD4 cells prior to adoptive transfer would
improve the ability of HA specific Th1s and CD8s to eradicate AB1HA tumours, mice
were depleted of CD4 cells with 4ug of GK1.5 given intravenously, followed 24 hours
later by adoptive transfer of CL4 CD8 T cells with or without in vitro differentiated HNT
Th1s. This experiment was initially performed in the prophylactic setting and survival
was compared to mice that did not receive CD4 depletion (Figure 7-2A). This showed
that in the prophylactic treatment setting, removing endogenous CD4 cells did not
improve the ability of mice to reject tumours. In mice receiving Th1s and CL4s, fewer
mice rejected tumours when endogenous CD4s were removed, although this was not
significant (p=0.24).

This experiment was then performed in a therapeutic treatment setting, where mice
with established AB1-HA tumours were treated with gemcitabine on day 9 (Figure
7-2B). Some groups of mice received adoptive transfer of HA specific T cells 24 hours
after gemcitabine. In addition, some mice were depleted of endogenous CD4 cells
prior to treatment where indicated.

Compared to mice that were treated with gemcitabine alone, removal of CD4s prior to
gemcitabine treatment resulted in a non-significant trend towards increased survival
(p=0.063), with around 20% of mice surviving long term. Survival was also improved by
treatment with gemcitabine followed by adoptive transfer of HA specific CD8s (p=0.04,
compared to gemcitabine only group) and with gemcitabine followed by Th1s + CD8s (p=0.13, compared to gemcitabine only).

However, when CD4 depletion was combined with adoptive transfer, a highly significant improvement in survival was observed. Compared to mice that received gemcitabine and adoptive transfer alone, depletion of CD4 cells prior to treatment improved survival in mice receiving HA specific CD8s (10 of 18 survivors; p=0.0065) and in mice receiving Th1s and CD8s (11 of 18 survivors; p=0.0004).

Amongst mice treated with CD4 depletion, gemcitabine and adoptive transfer, there was no difference in survival between mice receiving HA specific CD8s and mice receiving Th1s and CD8s (p=0.61), indicating that in established tumours, it is the removal of endogenous CD4s combined with the addition of HA specific CD8s which accounts for the majority of the effect seen. In contrast to the prophylactic treatment setting, the Th1s do not appear to be contributing to the ability of mice to reject tumours in the gemcitabine treatment setting.
The effects of CD4 depletion prior to treatment in prophylactic and gemcitabine treatment setting

A  Mice received adoptive transfer of $5 \times 10^5$ CL4 CD8 T cells with $1 \times 10^6$ in vitro differentiated HNT Th1s where indicated and were challenged with $5 \times 10^5$ AB1-HA cells subcutaneously. CD4 cells were depleted from groups as indicated the day prior to adoptive transfer through intravenous injection of 4 ug of GK1.5 antibody. Mice were culled when tumours reached 100 mm$^2$. N=6, experiment performed once. B  Mice with 9 day old AB1-HA tumours were treated with 240 ug/g gemcitabine i.p. and 24 hours later received adoptive transfer of $5 \times 10^5$ CL4 CD8 T cells with $1 \times 10^5$ HNT Th1 cells where indicated. Some groups of mice were also depleted of CD4 cells 1 day prior to gemcitabine by intravenous injection of 4 ug of GK1.5 antibody. * denotes significance by logrank test (* p<0.05, ** p<0.01, *** p<0.001). Combined results of three independent experiments shown with total between 6-18 mice per group.
7.2.3 Depletion of endogenous CD4 cells prior to treatment, does not improve survival of Th1s adoptively transferred following gemcitabine

In order to examine whether the improved survival described above was reflected in an improved ability of HA specific CD4 cells to provide cognate help to HA specific CD8 T cells, concurrent to the above experiment, groups of mice were treated with CL4 CD8s or CL4 CD8s plus HNT Th1s, with or without the prior depletion of endogenous CD4 cells. The effects of HA specific Th1 help on recovery of thy1.1+ CL4 CD8s 7 days after adoptive transfer was determined in both the post gemcitabine setting and in mice that did not receive gemcitabine. In order to have tumours of a sufficient size to yield adequate numbers of cells for analysis, gemcitabine treated mice were treated at day 14, when tumours were larger than that seen in the growth experiments. To enable adoptively transferred cells to be injected on the same day and harvests to be performed on the same day, tumour inoculations were staggered between gemcitabine treated mice and non-gemcitabine treated mice (Figure 7-3A).

Recovered thy1.1+ve CD4 cells were identified by flow cytometry and numbers of cells in DLN, spleen and tumour analysed (Figure 7-3B). Depletion of endogenous CD4 cells prior to adoptive transfer did not significantly alter recovery of thy1.1+ CD4 cells in untreated mice from the DLN, spleen or tumour. As noted in chapter 6, survival of adoptively transferred CD4 cells in all compartments was impaired in mice treated with gemcitabine, compared with untreated mice, but this was only significant in the spleen (p=0.04). Depletion of endogenous CD4s prior to gemcitabine treatment did not significantly improve recovery of thy1.1+ CD4s from the DLN, spleen or tumour.
Overall these results indicated that recovery of thy1.1+ CD4s was reduced in the post-gemcitabine setting, and that depletion of endogenous CD4s prior to treatment did not significantly improve the survival of adoptively transferred Th1s.
Figure 7-3  Recovery of adoptively transferred HA specific thy1.1+ CD4 T cells 7 days after adoptive transfer

A  Groups of mice were either treated with gemcitabine on day 14 or were not treated. All mice received adoptive transfer of 5x10^5 CL4 CD8 T cells with or without 1x10^6 HNT Th1s. Adoptive transfer was performed on day 10 after AB1HA inoculation for untreated mice and day 15 for gemcitabine treated mice. Some groups were depleted of CD4 cells by intravenous injection of 4ug of GK1.5 antibody 24 hours prior to treatment as indicated. Harvests were performed 7 days after adoptive transfer and recovered thy1.1+ve cells identified by flow cytometry.

B  Numbers of recovered thy1.1+ve CD4 cells in DLN, spleen and tumour in mice treated with CD4 depletion and gemcitabine. Tumour values expressed as percentage of total tumour cells. N=3 per group.
7.2.4 HA specific Th1s do not help CD8s infiltrate tumours following gemcitabine treatment

The effects of endogenous CD4 depletion and addition of HA specific Th1s on recovery of HA specific CD8 T cells 7 days after adoptive transfer was determined in mice treated as described in Figure 7-3. Thy1.1+ CD8 T cells were identified in cells recovered from the DLN, spleen and tumour by flow cytometry. In mice that were not treated with gemcitabine, neither CD4 depletion, Th1 transfer nor a combination of the two made any difference to the numbers of thy1.1+ CD8 T cells found in the DLN or spleen (Figure 7-4). Tumour infiltration of thy1.1+ CD8s was not altered by either depletion of endogenous CD4s or transfer of HA specific Th1s alone. However, if endogenous CD4 depletion and transfer of HA specific Th1s were both performed, then thy1.1+ve CD8 tumour infiltration was significantly increased, suggesting that the Th1s were able to help CD8s infiltrate tumours but only if endogenous CD4s were removed first.

In the post gemcitabine setting this effect was not observed. Although total numbers of thy1.1+ve CD8s were generally higher in the DLN in gemcitabine treated mice than in non-gemcitabine treated mice, no improvement in thy1.1+ CD8 recovery was seen with CD4 depletion or Th1 transfer in either the DLN, spleen or tumour (Figure 7-4).
Figure 7-4  Recovery of adoptively transferred HA specific thy1.1+ CD8 T cells 7 days after adoptive transfer

Groups of mice were treated according to the schedule in A, as described in Figure 7-3, Tumour inoculations were staggered between mice treated with gemcitabine or not to ensure tumour sizes were equal at the time of harvest. B Numbers of recovered thy1.1+ve CD8s were determined by flow cytometry. Tumour values expressed as a percentage of total cells. 3 mice per group. * p<0.05 by students t test.
The observation that HA specific CD8 tumour infiltration in mice not treated with gemcitabine was increased by co-transfer of Th1s, suggested that Th1s had the potential to help CD8s infiltrate tumours. The lack of help given by Th1s to CD8s in the post gemcitabine setting could have been explained by the reduced survival of Th1s transferred following gemcitabine, as described in section 6.2.7. Therefore, when this experiment was repeated, an increased number of Th1s were transferred in an attempt to compensate for this loss and see if the effects seen in the untreated setting could be replicated in the post gemcitabine setting.

Mice were again treated according to the schedule described in Figure 7-3A, but received a threefold increased number of Th1s. When thy1.1+ CD4 cells were recovered from mice 7 days after adoptive transfer, the number of thy1.1+ CD4 cells present in the DLN, spleen and tumour (Figure 7-5A) had increased from that seen in the original experiment (Figure 7-3B), consistent with the increased numbers of cells injected. However, despite the increased numbers of Th1 CD4 cells present, no effects of Th1s on CD8 recovery from DLN, spleen or tumour were observed in mice treated with or without gemcitabine, or in mice depleted of CD4s prior to transfer (Figure 7-5B). The previously observed positive effects on CD8 tumour infiltration of combined CD4 depletion and Th1 transfer in the non gemcitabine treated setting was not repeatable.

Thus, there was no consistent evidence that in vitro differentiated HA specific Th1 cells were able to provide any help to HA specific CD8 cells in either the untreated or gemcitabine treated setting, with or without prior CD4 depletion.
Figure 7-5  Repeat of experiment described in Figure 7-3 and Figure 7-4, with transfer of increased numbers of Th1s

Mice were treated as described in Figure 7-3A, but received $3 \times 10^6$ Th1s alongside $5 \times 10^5$ CL4 CD8s. Recovery of Thy1.1+CD4+ cells and Thy1.1+CD8+ cells 7 days after transfer is shown in A and B respectively. 3 mice per group.
7.2.5 Transfer of in vitro differentiated T regs abrogates the beneficial effects of CD4 depletion followed by adoptive transfer of HA specific T cells

Although CD4 depletion did not improve the capacity of HA specific Th1s to help HA specific CD8s expand or infiltrate tumours, it was evident that CD4 depletion in combination with adoptive transfer of HA specific CD8s did significantly improve survival in the context of gemcitabine treatment (Figure 7-2B). I hypothesised that removal of established regulatory CD4 cells was the mechanism by which CD4 depletion improved anti-tumour responses. To confirm that it was removal of the Treg subset which was critical to this response, I looked to see if I could abrogate this effect by adoptively transferring in vitro differentiated T regs alongside the Th1s and CD8s. Mice bearing established AB1HA tumours received CD4 depletion on day 8 and were treated with gemcitabine on day 9. Adoptive transfer of CL4 CD8s and HNT Th1s was performed on day 10 with one group of mice also receiving an equal number of in vitro differentiated HNT Tregs.

In those groups receiving adoptive transfer of CD8s and those receiving Th1s and CD8s, a high proportion of mice rejected tumour (Figure 7-6). However the adoptive transfer of HA specific Tregs alongside HA specific CD8s and Th1s completely abrogated the beneficial effect of CD4 depletion and adoptive transfer (p= 0.02). The survival curve of this group was no different to that of mice treated with gemcitabine alone, demonstrating that the anti-tumour activity of HA specific CD8s is suppressed by Tregs and that the beneficial effect of CD4 depletion is mediated through removal of endogenous Tregs.
Figure 7-6  Adoptive transfer of in vitro differentiated Tregs abrogates the effects of CD4 depletion and adoptive transfer of HA specific effector T cells

Groups of AB1-HA tumour bearing mice were treated on day 9 with 240ug/g gemcitabine. Some groups were depleted of CD4 cells on day 8 where indicated. Adoptive transfer was performed on day 10. Mice received $5 \times 10^5$ CL4 CD8 cells alongside $1 \times 10^6$ HNT Th1s where indicated. One group also received adoptive transfer of $1 \times 10^6$ in vitro differentiated HA specific Tregs. Survival curves analysed by log rank test and * denotes p<0.05. Experiment performed once, N=6 per group.
7.3 Discussion

The experiments described in this chapter were performed because results from chapter 5 suggested that suppression by regulatory CD4 T cells could be limiting the anti-tumour activity of adoptively transferred tumour specific T cells. Additionally, in chapter 6 I demonstrated that in vitro differentiated Th1 cells do not expand in vivo when transferred in the post gemcitabine setting, and I hypothesised that this could be due to competition from proliferating endogenous T cells. For both of these reasons, I hypothesised that increasing the depth of the CD4 depletion prior to treatment would improve the ability of the adoptively transferred cells to eradicate tumours.

Through the use of a very low dose of CD4 depleting antibody, it was possible to remove nearly all circulating CD4 cells without adversely affecting the CD4 cells adoptively transferred 24 hours later. This technique was dependent on titrating the amount of antibody to just below the saturating dose, so that there was no free antibody available to deplete the CD4 cells adoptively transferred 24 hours later. It is striking that the dose required to achieve this effect is several fold lower than that commonly used elsewhere (Nowak, Robinson et al. 2003; Teng, Swann et al. 2010). Although I did not examine whether this dose depleted CD4 cells within the tumour, depletion of CD4 cells in the lymph nodes and spleen was seen, indicating that removal of CD4 mediated suppression of CD8 priming was potentially possible. Others have shown that dose response curves for GK1.5 are equivalent for spleen, lymph node and blood but higher doses are required to deplete CD4 cells from the thymus (Ghobrial, Boublik et al. 1989).
I found that removal of endogenous CD4 cells prior to treatment did not improve the ability of HA specific T cells to reject AB1HA tumours in the prophylactic setting. In naive mice, immune cells suppressive to anti-tumour responses have not had a chance to develop, so this finding is not unexpected. However it does contrast with my findings in chapter 3 and with the findings of others (Teng, Swann et al. 2010), where prophylactic CD4 depletion did mediate tumour rejection. However these experiments did not involve adoptive transfer of effector T cells prior to inoculation. Darasse-Jeze showed that if effector T cells were present at the time of tumour inoculation then Tregs were ineffective at suppressing immune responses, implying Treg depletion may not have added benefit in such a setting (Darrasse-Jeze, Bergot et al. 2009). This is of relevance to the prophylactic model described here, where mice received HA specific Th1s and CD8s prior to tumour inoculation, and could explain why Tregs are not limiting in such a situation.

Another mechanism by which lymphodepletion may enhance the activity of adoptively transferred cells is through augmenting homeostatic proliferation (Muranski and Restifo 2009). However these findings suggest that, at least in the prophylactic situation, the activity of adoptively transferred Th1s is not enhanced by the provision of more ‘space’ for expansion to occur.

In contrast to the prophylactic setting, I found that removal of endogenous CD4 cells prior to gemcitabine and adoptive transfer significantly increased survival in mice receiving HA specific T cells. This suggested that, in established tumours, inhibitory CD4s were suppressing effector T cells responses and that the lymphodepletion induced by gemcitabine was insufficient to remove this suppression. In the absence of adoptive transfer of HA specific T cells, there was a trend towards improved survival.
when CD4s were depleted prior to gemcitabine but only a small proportion of mice were cured. Thus removal of suppressive CD4s prior to gemcitabine was not in itself sufficient to mediate tumour rejection, which still required the addition of effector T cells.

However, there was no added benefit of co-transfer of Th1s and CD8s over CD8 transfer alone in mice treated with CD4 depletion and gemcitabine. This contrasts with the prophylactic experiments where cures were only seen in mice receiving both Th1s and CD8s. This demonstrates that removal of suppressive CD4 cells is critical to achieving CD8 mediated tumour eradication of gemcitabine treated established tumours, but that the addition of Th1 polarised helper CD4 cells is not of added benefit in such a setting.

The inability of Th1s to help CD8s eradicate established, gemcitabine treated tumours is reflected in the lack of effects of Th1 co-transfer on expansion or infiltration of HA specific CD8s in the DLN, spleen or tumour. In chapter 6, it was observed that in vitro differentiated Th1 cells transferred after gemcitabine did not expand between days 2 and 7 after transfer, which contrasted with what was seen in untreated mice. In the experiments described in this chapter, numbers of recovered thy1.1+ CD4 cells were also reduced at day 7 compared with mice that did not receive gemcitabine. Depletion of endogenous CD4 cells did not significantly improve the recovery of transferred Th1 cells in the DLN, spleen or tumour. Therefore the limited help provided by the adoptively transferred Th1s to CD8s in these experiments could be explained in part by the detrimental effects of the post gemcitabine environment on their survival. However, increasing the number of transferred Th1s did not improve their capacity to help CD8s. Furthermore, in mice not treated with gemcitabine I was similarly unable
to consistently demonstrate that CD8 expansion or tumour infiltration had been affected by the provision of Th1 help. This suggests that the reduced survival of Th1s in the post gemcitabine setting does not fully explain why Th1s did not provide effective help to CD8s in this situation.

The mechanism by which CD4 depletion improves survival in mice treated with gemcitabine and CD8 adoptive transfer was examined. When in vitro differentiated HA specific Tregs were adoptively transferred alongside Th1s and CD8s, the beneficial effects of CD4 depletion and adoptive transfer were completely abrogated. This confirmed that it was the depletion of the established CD4 Treg subset which explained the improved survival seen following CD4 depletion.

Although the growth experiments suggested that CD4 depletion was necessary to achieve cures with adoptive transfer of HA specific CD8s, when cells were harvested 7 days after transfer, I was unable to show that removing established CD4s improved expansion or tumour infiltration of HA specific CD8s. Although Treg depletion can promote non-CD8 mediated mechanisms of tumour rejection, CD8s were found to be essential mediators of tumour rejection following Treg depletion (Teng, Swann et al. 2010), so it would be surprising if CD8s were not involved in this response. It is possible that endogenous CD4 depletion promotes activity of endogenous CD8s, resulting in an additive rather than synergistic effect with the adoptively transferred HA specific CD8s. It is also important to note that in order to harvest sufficient cells from tumours for analysis, it is necessary to use tumours which are larger at the time of treatment than used in the growth experiments. This approach relies on the presumption that adoptively transferred T cells will behave in the same way in small and large tumours, which may not be the case. In addition, I only performed harvests
7 days after adoptive transfer, it is possible that using an earlier time point may have shown different results.

In summary the results in this chapter show that:

- Established, endogenous regulatory T cells limit the activity of adoptively transferred HA specific CD8s and removal of Tregs is critical to achieving a high proportion of cures with chemotherapy followed by adoptive CD8 immunotherapy.
- Lymphodepletion induced by gemcitabine chemotherapy is insufficient on its own to remove Treg mediated immunosuppression.
- HA specific, in vitro differentiated Th1 helper CD4s do not help HA specific CD8s to expand or infiltrate established tumours and do not have anti-tumour activity against gemcitabine treated tumours.
8 Final Discussion
At the start of this project I hypothesised that:

- The balance between different effector and suppressive CD4 subsets determines tumour rejection or tolerance
- Adoptive transfer of *in vitro* differentiated tumour specific CD4 subsets could promote tumour rejection
- The post chemotherapy environment presents a favourable window for adoptive cell transfer

To investigate these hypotheses I had to establish a model with which to investigate tumour antigen specific T cell immune responses, specifically looking at the different roles that CD4 subtypes play in anti-tumour responses. The aims of this project were:

- To establish protocols for *in vitro* differentiation of tumour specific Th1s, Th2s, Th17s, Tregs
- To investigate the *in vivo* activity of *in vitro* differentiated tumour antigen specific CD4 cells against tumours treated with cytotoxic chemotherapy
- To investigate the effects of cognate help provided by HA specific CD4 subsets on HA specific CD8 T cells

In this chapter I will review all the results obtained with reference to these hypotheses and aims. In instances where the results do not allow conclusions to be drawn but instead have generated further hypotheses, I shall suggest additional experiments which could be considered to investigate the questions raised by this project.
The balance between CD4 subsets determines tumour rejection/tolerance?

The initial premise behind this project was that CD4 T cells play an important role in determining the immunological outcome to a tumour. This premise is supported by a growing body of literature which has shown that effector CD4 cells promote CD8 and other anti-tumour immune responses and, conversely, that removing regulatory CD4 T cells can lead to tumour rejection (discussed in section 1). When I depleted CD4 cells from mice during treatment with repeated doses of gemcitabine followed by anti-CD40 antibody, I found that, although the time to tumour outgrowth was increased by the removal of CD4 T cells, there was no difference in the number of mice cured of tumour. This suggests that CD4 cells may be having some anti-tumour effects but did not imply that they were of crucial importance to final outcome in this setting. It is possible that the effects of CD4 cells during this treatment regimen are blunted through the repeated dosing schedule or because anti-CD40 replaces one of the main mechanisms of CD4 help.

When CD4 cells were depleted from mice prior to a single dose of gemcitabine, followed by adoptive transfer of tumour antigen specific CD8s there was a significant improvement in the ability of mice to reject established tumours. This was due to removal of suppression by established regulatory T cells. In chapter 3 depletion of CD4 cells during treatment with gemcitabine and anti-CD40 increased CD8 activation and in chapter 5 it was seen that adoptively transferred Tregs trafficked to the tDLN and reduced HA specific CD8 tumour infiltration, suggesting that regulatory CD4s inhibit CD8 responses, and that this was likely to occur at the level of CD8 priming in the DLN.

In addition, CD4 T cells appeared to have a different influence on anti-tumour responses during different periods of tumour emergence and growth. In mice
subsequently treated with gemcitabine and anti-CD40, depletion of CD4s prior to
tumour inoculation delayed tumour growth and improved survival times, which is
consistent with other similar studies (Teng, Swann et al. 2010) and may reflect the
observation that the regulatory CD4 T cell response is most rapidly established on first
exposure to tumour antigen (Darrasse-Jeze, Bergot et al. 2009)resulting in an overall
negative impact from CD4s on anti-tumour responses at this early stage. Rejection of
tumour in the prophylactic setting only occurred when tumour specific Th1s were
adoptively transferred alongside CD8s. This was not affected by prior depletion of CD4
cells. These results suggest that on first exposure to tumour antigen it is the presence
or absence of effector, Th1 polarised antigen specific CD4s which determines whether
tumour rejection occurs.

In contrast, in established gemcitabine treated tumours, rejection only occurred when
regulatory CD4s were depleted prior to adoptive transfer of tumour specific CD8s. This
was not affected by the additional provision of Th1 help, suggesting that once a
tumour has established then it is the presence or absence of regulatory CD4 cells,
rather than effector CD4 cells, which determines whether CD8s can eradicate tumours.

Adoptive transfer of in vitro differentiated tumour specific CD4 subsets will mediate
tumour rejection

Tumour rejection mediated through adoptive transfer of tumour antigen specific Th1s,
in conjunction with CD8s, was observed in the prophylactic situation, demonstrating
the potential for Th1 polarised CD4 cells to help eradicate AB1-HA tumour cells.
However this result was not replicated in a more clinically relevant scenario, when
mice with established tumours were treated with gemcitabine followed by adoptive
cell transfer. Thus once a tumour was established I was unable to demonstrate any positive anti-tumour effects from adoptive transfer of in vitro polarised CD4 cells.

The reasons why CD4 cells did not have any effects on tumours beyond the prophylactic setting are discussed later in this chapter.

**The post chemotherapy environment provides a favourable window for adoptive transfer**

Cytotoxic chemotherapy can synergise with immunotherapy through a number of mechanisms, including increased tumour antigen release, inducing immunogenic tumour cell death, depletion of immunosuppressive cells and promoting homeostatic proliferation (discussed in section 1.4). For these reasons I hypothesised that the post chemotherapy environment would provide a favourable environment for adoptive transfer of tumour specific CD4 T cells, and thus provide a window of opportunity to redirect the immune response away from tolerance and towards rejection.

The results described herein do not support this hypothesis. Not only did adoptive transfer of CD4 cells in the post chemotherapy environment have no effect on the ability of mice to reject tumours, but surprisingly the survival of in vitro differentiated Th1 cells was adversely affected by the post gemcitabine environment compared to cells injected into untreated tumour bearing mice.

The reduced persistence of Th1s in the post gemcitabine environment was not due to direct cytotoxicity on the transferred cells but instead the population of cells failed to expand between day 2 and 7 after transfer. The reasons for this are not clear. If we consider the immune-modulating effects of chemotherapy outlined above, it is
possible that increased release of tumour antigen and increased antigen presentation could provoke apoptosis in cells which are vulnerable to activation induced cell death. Additionally, since the cells were injected during a period of lymphopenia then IL-7 driven homeostatic proliferation is likely to have been the main driver of restoration of total T cell numbers. In vitro differentiated Th1 cells express low levels of the IL-7 receptor, potentially putting them at a disadvantage in such a setting.

However, increasing the depth of the CD4 depletion using a CD4 depleting antibody did not improve the persistence of the adoptively transferred cells in the post gemcitabine environment, suggesting that competition from proliferating endogenous lymphocytes for cytokines may not be the only reason why gemcitabine was detrimental to survival of adoptively transferred CD4 cells. Others have shown that CD8 T cells expanded in vitro in IL-2 underwent apoptosis without continuous IL-2 supplementation and survived poorly in vivo in the absence of exogenous IL-2 provision (Yee, Thompson et al. 2002; Mueller, Schweier et al. 2008). Therefore Th1 cells expanded in vitro in IL-2, which do not themselves produce IL-2, may also be dependent on IL-2 produced by other T cells for survival. Thus lymphodepletion by gemcitabine could reduce the amount of IL-2 available for the transferred Th1s. It is noteworthy that most adoptive cell therapy protocols involve the administration of large quantities of exogenous IL-2 following cell transfer (Dudley 2011).

8.1 Why don’t antigen specific CD4 helper T cells provide cognate help to CD8s in this model?
Although Th1s were observed to have anti-tumour activity in the prophylactic treatment model, this was not observed in treatment of established tumours. This may have been partly due to the negative effects of gemcitabine on their persistence in vivo. However, when I performed harvests and tracked the behaviour of the transferred cells in vivo, it was clear that co-transfer of HA specific Th1s had no consistent impact on HA specific CD8 expansion or tumour infiltration in either gemcitabine treated mice or in untreated mice. One of the aims of this model was to examine the effects of cognate help from CD4s on CD8s and hence provide some mechanistic data behind any treatment effects observed. Previous work with this model has shown that transfer of naive HA specific CD4s alongside HA specific CD8s at the time of tumour inoculation increased survival of the CD8s and their ability to infiltrate the tumour site and eradicate tumours (Marzo, Kinnear et al. 2000). It is important to consider why no such effects were seen in the experiments described.

**Problems with the model?**

In the absence of a clear positive control demonstrating that HNT CD4 cells have the capacity to help CL4 CD8s in vivo, it is important to consider first whether there may be a problem with the HA-transgenic system utilised. The AB1-HA transgenic model relies on a number of factors: 1) HA is expressed by tumour cells; 2) HA protein can be assimilated and processed by antigen presenting cells and epitopes presented on MHC class I and class II; 3) The T cell receptor on HNT CD4 cells can bind to HA epitopes presented on MHC class II; 4) The T cell receptor on CL4 CD8 T cells can bind to HA epitopes presented on MHC class I; 5) Upon ligation of their TCR, HNT and CL4 lymphocytes display normal effector functions.
The expression of HA by AB1-HA cell lines was confirmed by RT-PCR within the lab on several occasions. It would be important to repeat this on tumours harvested ex vivo, especially those which have not responded to HA targeted treatments, to ensure HA expression is not being lost in vivo. However, the observation that HA specific Th1s and HA specific CD8s, are activated and expand in vivo in the presence of AB1-HA tumour, and are recoverable from AB1-HA tumours and the tumour DLN at a high frequency suggests that HA expression is being maintained, that HA protein is being presented by antigen presenting cells and that HNT and CL4 lymphocytes can recognise HA epitopes presented on MHC. Repeating this experiment with a control group using the AB1 parent line would confirm that this was an HA specific response.

In addition, I confirmed that in vitro differentiated HNT CD4 cells were able to respond in vitro to splenocytes loaded with HNT peptide and it has been confirmed that CL4 CD8 cells are able to respond to CL4 peptide (Amanda Cleaver – unpublished data). Repeating this experiment with APCs loaded with HA protein and/or AB1-HA tumour lysate would demonstrate that APCs were able to process and present HA protein effectively in vitro.

Finally the model requires that HNT and CL4 lymphocytes function normally, that is in the same way as the parent balb/c lymphocytes. Although these cells did respond in vitro to peptide stimulation, to test whether HNT CD4s are able to help CL4 CD8s in vivo, an adoptive transfer experiment into RAG knockout mice lacking T cells could be performed in conjunction with an HA stimulus, such as AB1-HA tumour challenge, influenza inoculation or an HA directed vaccine. The magnitude of the CL4 CD8 response in the presence or absence of HNT CD4s or wildtype CD4s could then be determined.
Assuming all the above criteria have been satisfied, it is still necessary to look for the effects of CD4 help on HA specific CD8s at a time and location where differences are detectable. Since mice cured of tumour do not have tumours available for harvest, it was necessary to use larger tumours for the harvest experiments than those used in the treatment experiments. The lack of effects of the adoptively transferred CD4s in the harvest experiments, could therefore have been confounded by different tumour sizes. In addition, I chose to perform harvests 7 days after adoptive transfer, as this was around the time when tumours were seen to regress in the treatment experiments. However, it is not certain that this is the optimal time to observe the peak CD8 response, especially as tumour sizes were not equivalent between the treatment and harvest experiments.

**What kind of CD4 cell is generated through in vitro differentiation?**

It is apparent from the results presented in chapter 4 that the viability and phenotype of in vitro differentiated CD4 cells are highly affected by the precise culture conditions employed, including the duration of the activation stimulus, the polarising cytokines, the type of media and the addition of exogenous IL-2. The conditions needed to produce polarised cells at a high viability differed between subsets, with continual TCR stimulus necessary to maintain Th17 differentiation but being detrimental to Th1, Th2 and Treg viability. Furthermore, by shortening the duration of the activation stimulus and adding IL-7 instead of IL-2 to cultures, it was possible to produce ‘Th1’ cells, which had markedly different levels of IL-2 and CD62L expression from those produced using the original protocol, despite the expression of similar levels of IFNg. The in vivo behaviour of these cells following adoptive transfer differed accordingly. Thus
comparisons between different models which have used different methods to polarise CD4 cells need to be made cautiously.

I found that Th1s were the only CD4 subset with anti-tumour activity in this model. Although Th1 responses have long been known to be important in tumour immunology, recent work has suggested that in vitro differentiated Th17s elicit more effective anti-tumour responses than Th1s following adoptive transfer into C57BL/6 mice (Muranski, Boni et al. 2008; Martin-Orozco, Muranski et al. 2009). However, I was unable to find evidence that Th17s survived in vivo after adoptive transfer in the model used here. It is unclear why this is the case but it could be due to differences in the model used in these studies, including the mouse strains, the use of a lymphoablating preconditioning regimen and different differentiation protocols.

Wrong place, wrong time?

Not only does the function of in vitro differentiated CD4 cells depend on their differentiation phenotype but will also depend on the locations that these cells track to after adoptive transfer. Following injection, in vitro differentiated CD4 subsets display differing capacity to track to lymphoid organs and to the tumour. In naive mice Th1s and Tregs possessed the greatest capacity to home to lymph nodes, and this is reflected in their high expression of CD62L. In the presence of tumour however, Tregs accumulated in the draining node substantially faster than Th1s. However, Tregs disappeared by day 7 and were not found in the tumour site. This suggests that the suppressive effects of in vitro differentiated Tregs are likely to be mediated at the level of the lymph node in the first few days after transfer.
This is of relevance to the model we chose to use in this project. We co-transferred naive HA specific CD8s alongside the CD4 cells, in order to ensure adequate numbers of CD8 effectors were available for the CD4 helper cells to help. Naive CD8s express high levels of CD62L and accumulate readily in the DLN immediately after transfer. For the HA specific CD8 response to be initiated, these cells need to be activated in the DLN, meaning that priming of naive HA specific CD8s in the DLN is the first rate limiting step in this process. Thus, this model is likely to be sensitive to interventions at the level of CD8 priming and hence the effectiveness of CD4 help is likely to be dependent on whether CD4 helper cells are present in the DLN at this early stage during CD8 priming. Thus, discordant initial tracking to the DLN of the CD4 effector subsets and the naive, HA specific CD8s could explain why we did not see any effect of CD4 help by Th1s on the CD8s, whereas we were able to observe a negative impact from transfer of Tregs.

**Does in vitro differentiation produce Th1 effectors, not helpers?**

These observations raise the question of what type of CD4 cell in vitro differentiation actually generates and how closely do they represent their in vivo counterparts. CD4 subsets are commonly identified by the secretion of their ‘signature’ cytokine - IFNg for Th1s, IL-4 for Th2s, IL-17 for Th17s – or by expression of the transcription factor foxp3 for Tregs. However CD4 cells perform multiple functions in addition to secretion of these cytokines. Hence the true functional capability of CD4 cells may not be reflected by measurement of just one phenotypic marker. When in vitro differentiated CD4 cells were analysed for expression of other markers associated with CD4 helper functions, CD40L and IL-2, it was clear that some subsets lost expression of
these markers during in vitro culture. Th1s were the only subset to maintain expression of CD40L during in vitro culture, however this was not detectable on Th1s ex vivo, two days after adoptive transfer. Secretion of IL-2 was lost by Th1s and Th2s after the first two days of in vitro culture, at the point that they began to express IFNg and IL-4 respectively.

I hypothesised that, in the case of Th1s, 5 days of in vitro culture produced Th1 cells with an effector phenotype, rather than a helper phenotype. It also appeared that during differentiation Th1 cells may be moving through a helper phase, characterised by high expression of IL-2 and CD40L, into an effector phase, characterised by high expression of IFNg. Whether these in vitro observations reflect events during in vivo differentiation of CD4 cells is not clear. Villarino et al proposed a similar model and also suggested that the dynamics of IL-2 and IFNg expression may correlate with migration of cells from lymph nodes into effector site tissue (Villarino, Stumhofer et al. 2006). Interestingly, I observed that the endogenous CD4 cells isolated from the DLN of tumour bearing mice produce large amounts of IL2 and little IFNg, whereas CD4 cells isolated from tumours produce large amounts of IFNg and little IL-2. Thus the secretion profile of IFNg and IL-2 by Th1 cells at the end of in vitro differentiation resembled endogenous CD4 cells found in the effector site and not at the site of priming.

Since Th1s were found to be effective in the prophylactic treatment setting, it is clear that they do possess some anti-tumour activity. In the absence of any demonstrable effects on HA specific CD8s it is possible that these cells are acting through non-CD8 related mechanisms. CD4 cells can exhibit direct effector function against tumour cells or can recruit other immune cells such as macrophages, granulocytes or NK cells
(discussed in section 1.2.3). Thus the adoptively transferred Th1s and the CD8s may be acting independently of one another and the survival advantage seen with transfer of Th1s and CD8s together in the prophylactic situation could be an additive rather than a synergistic effect. Repeating the prophylactic treatment experiment with transfer of Th1s alone would demonstrate whether their effect is independent of HA specific CD8s. Depletion of the endogenous CD8s could then be performed using a CD8 depleting antibody to determine whether Th1s acted entirely through non-CD8 mechanisms.

**Endogenous immune cells may be confounding the effects of HA specific adoptively transferred cells**

It is important to consider the influence the endogenous immune system has on the activity of the transferred HA specific cells and the influence this may be having on assays which look at the transferred HA specific cells only. Depletion of endogenous CD4 cells prior to adoptive transfer clearly indicated that endogenous regulatory CD4s were suppressing the ability of HA specific CD8 cells to eradicate established tumours. When HA specific Tregs were transferred back in these effects were abrogated. Repeating this experiment with Tregs generated from wild type balb/c mice would enable us to determine whether the suppressive effects of these Tregs were dependent on HA antigen recognition.

I assumed that HA specific CD4s would be able to provide cognate help for HA specific CD8s. However, presuming this to be true, this still does not preclude the possibility that HA specific CD8s may also be able to receive help from endogenous CD4 cells, whether they are cognate for HA or for other tumour antigens co-expressed by AB1-
HA. One explanation for the lack of benefit from transfer of HA specific Th1s in established tumours is that adequate CD4 help for the HA specific CD8s is already available from endogenous helper CD4 cells, albeit this is only unmasked when overriding suppression from regulatory CD4s is removed. This would contrast to the prophylactic situation where the recipient mouse is naïve to tumour antigens and hence the transfer of tumour antigen specific CD4 helper cells does provide help which is not otherwise available.

Thus it is possible that interactions between endogenous immune cells, both suppressive and effector, and the transferred HA specific T cells could be interfering with the ability to detect interactions between the HA specific CD4s and the CD8s.

### 8.2 Conclusions

In summary I developed a model with which to study the effects of different subtypes of tumour specific CD4 cell on tumour eradication by CD8 T cells in conjunction with cytotoxic chemotherapy. Four main conclusions can be drawn from these results.

1) The phenotype of in vitro differentiated CD4 subsets and their behaviour in vivo following adoptive transfer is highly dependent on the in vitro culture conditions.

2) Th1s were the only subtype with anti tumour activity on this model, but only when injected prior to tumour inoculation. The protocols utilised in this project produced Th1 cells which displayed an effector rather than a helper phenotype and they did not provide cognate help to CD8s.
3) Gemcitabine does not provide a favourable window for adoptive transfer of in vitro differentiated CD4 cells. Persistence of these cells in vivo is reduced in the post gemcitabine environment through mechanisms other than direct cytotoxicity on the transferred cells.

4) The ability of tumour specific CD8s to eradicate established tumours following gemcitabine is suppressed by endogenous regulatory T cells. Removal of Tregs is critical to achieving cures with chemotherapy and adoptive CD8 transfer.

These results provide a rationale for trying to combine different immunotherapies alongside conventional cytotoxic chemotherapy. Combining chemotherapy with both the provision of a CD8 targeted stimulus and removal of CD4 mediated suppression should be further explored as a strategy to improve outcomes for patients with cancer.
9 References


Ghiringhelli, F., N. Larmonier, et al. (2004). "CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows
immunotherapy of established tumors to be curative." Eur J Immunol 34(2): 336-44.


to mediate tumor regression following systemic CD40 activation." Journal of Immunology 173(10): 5923-8.


